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IMMUNOLOGICAL DIFFERENCES BETWEEN STRAINS OF POLIOMYELITIC VIRUS.*

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ALL who have worked with experimental poliomyelitis in monkeys are agreed that an attack of the disease from which the animal recovers leaves an almost absolute degree of immunity. Even if the infection shows itself only by excitability and transient ataxia without paralysis the monkey is subsequently resistant even to intracerebral inoculation with a highly active virus. This has necessarily led to the view that while different strains of the poliomyelitis virus may show great differences in the intensity of their action on the central nervous system, yet they are immunologically identical. One might compare the different strains of virus with a series of diphtheria toxins inactivated to various degrees by treatment with formaldehyde. All retain the power to provoke a qualitatively identical immunity, but their pathogenic activity ranges from the maximal to complete innocuity.

In the course of work on the neutralizing power of convalescent human sera (Burnet and Macnamara, 1929) several exceptions to this rule of complete subsequent immunity were encountered. The experiments in question were concerned with the response of monkeys, which had been previously infected and paralysed, to a second intracerebral injection of a poliomyelitic virus of different origin. A few experiments on the neutralizing power of convalescent monkey serum for two distinct viruses were also carried out. The two viruses used were those described in our previous communication—a local virus of moderate activity derived from a child dying in the Children's Hospital, Melbourne, in February, 1928, and the highly virulent "mixed virus" strain from the Rockefeller Institute which we received through the courtesy of Dr. Flexner.

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The experiments have had to remain incomplete, but the trend of the results obtained is sufficiently definite to warrant a report. Brief protocols of these experiments may be grouped under three headings:

1. FAILURE OF A PREVIOUS INFECTION WITH LOCAL VIRUS TO PROTECT AGAINST ROCKEFELLER VIRUS.

Monkey 16.—29.1.29. Local virus and convalescent serum were injected intracerebrally. The animal showed no symptoms, and on 6.3.29 was given 0·5 c.c. of local virus alone by the same route. Paralysis of legs was evident on 14.3.29 and became complete. The arms and upper part of the body were unaffected, but almost complete paralysis of both legs with contractions of the hips in flexion remained permanent. On 18.7.29, 0·5 c.c. of a Rockefeller virus filtrate was injected intracerebrally. Five days later the monkey appeared to be sick and its movements were tremulous. This condition lasted till 28.7.29, when the animal became rapidly paralysed and prostrate. The cord was removed, and an emulsion injected intracerebrally into Monkey 56 on 1.10.29. On 7.10.29 this monkey became paralysed, with the typical complete prostration produced by the Rockefeller virus.

Monkey 58.—On 29.1.30 a mixture of local virus and immune human serum was injected, no symptoms being produced. The injection on 18.2.30 of 0·5 c.c. of local virus suspension was followed on 27.2.30 by typical paralysis, first in the right leg, then involving the other leg and right arm. All movements were very tremulous. The condition gradually improved, and by 3.7.30 both limbs on the left side were fairly normal, the right arm was weak, and there was contraction of the right ankle in dorsiflexion and general weakness of this limb. Another injection of local virus, 0·5 c.c., was made intracerebrally on this date without any resulting symptoms. The same suspension injected into Monkey 59 resulted in typical paralysis 12 days later.

On 5.8.30, 0·5 c.c. of a Rockefeller virus filtrate was injected. Five days later there was typical paralysis, with complete prostration and death on 11.8.30.

Monkey 64.—Local virus (0·5 c.c. of suspension) was injected intracerebrally on 5.8.30. On 13.8.30 paralysis of both legs was evident, becoming complete on the following day. There was no involvement of the upper half of the body. The legs remained almost completely paralysed, and by 3.9.30 contraction of the hips in flexion had occurred. On this date a few c.c. of blood were removed and the animal given 0·5 c.c. of a Rockefeller virus suspension intracerebrally. On 6.9.30 the monkey was abnormally excitable, and for the next 4 days was very tremulous in the movements of its arms. The condition was similar to that shown by Monkey 16, but in this case there was no increase in paralysis, and after 11.9.30 the animal appeared normal except for the old paralysis of its legs. The same suspension injected into a control monkey, 65, resulted in the usual paralysis on the 4th day going on to complete prostration within 24 hours.

Each of these three animals was presumably immune to the local virus after the first infection, but all showed some reaction to the second injection of Rockefeller virus. In one there was an apparently unmodified acute infection,

in another the onset of widespread paralysis was preceded by a period of five days of indefinite symptoms, and in the last the symptoms after the usual incubation period indicated an abortive infection without increase in the degree of paralysis.

2. THE NEUTRALIZING POWER OF SERUM FROM A MONKEY PREVIOUSLY PARALYSED BY LOCAL VIRUS.

Serum from Monkey 64 taken just before the second intracerebral injection was made, was tested for its power to inactivate the two viruses according to the usual technique. The results given in Table I show that in a dose of 0·5 c.c. the serum inactivates a local virus suspension but does not modify the infectivity of a Rockefeller virus filtrate.

TABLE I.

Monkey.	Virus.	Serum of Monkey 64, 3.9.30.	Result.
66	Rockefeller suspension	0·1 c.c.	Paralysed 4th day. Dead on 6th.
71	Rockefeller filtrate	0·5 c.c.	Paralysed 4th day. Dead on 5th.
68	Local suspension	0·1 c.c.	Paralysed 11th day. Dead on 12th.
73	Local suspension	0·5 c.c.	Survived without symptoms.

3. INFECTION BY A LOCAL VIRUS IN A MONKEY PREVIOUSLY PARALYSED BY PARTIALLY NEUTRALIZED ROCKEFELLER VIRUS.

Monkey 51.—On 8.8.29 received a mixture of Rockefeller virus filtrate and 0·1 c.c. of Victorian human immune serum (see previous paper, Table VIII). The monkey appeared quiet on the 7th and 8th days after inoculation, and on the 9th was holding the right arm in semiflexed position and not using it. It was very unsteady on its feet, tending to topple forwards. The weakness of gait improved rapidly, but partial paralysis and contracture of the right arm persisted. On 1.10.29, 0·5 c.c. local virus suspension was injected intracerebrally. The monkey was tremulous and weak in the legs on 7.10.29, and completely paralysed the following day.

Several attempts were made to obtain other survivors who had been paralysed with Rockefeller virus by injecting partially neutralized mixtures of virus and serum. Beyond a lengthening of the incubation period, however, it was found impossible to modify the infection short of its complete elimination. This agrees with the results of Fairbrother and Hurst (1930), who used the same virus for a large number of experiments and observed only one instance in which a monkey was paralysed but recovered. In this case the virus was administered together with serum from an immunized horse.

DISCUSSION.

The results of these experiments show clearly that paralysis by poliomyelitis is not necessarily indicative of complete resistance to subsequent infection, provided that the second infection is with a virus of greater virulence or different type from the first. The question arises as to whether the differences between viruses are sufficient to justify differentiation into immunological types, corresponding, for example, to the A and O types of foot and mouth disease. The evidence given above shows conclusively that infection and paralysis with our Australian virus usually does not protect against the American virus. Monkey 58 shows this particularly clearly. After being paralysed by local virus a subsequent injection of the same virus was without effect. A normal monkey used as a control showed typical poliomyelitis. The animal was therefore immune to the local virus, but the next injection with a Rockefeller virus filtrate showed that no resistance to this virus had been acquired. There was not even any increase in the incubation period of the disease.

Even in the one case (Monkey 64) that showed no increase in paralysis, there was fairly clear evidence that infection by the Rockefeller virus occurred at the usual time after injection, and the serum of this monkey had power to neutralize *in vitro* only the Victorian virus. Unfortunately the very high virulence of the Rockefeller virus, which in our hands has always caused paralysis on the 4th or 5th day with complete prostration and death within one or two days, has prevented attempts to test its power to immunize against the local virus. The one monkey available (Monkey 51) had only a moderate degree of paralysis after receiving a mixture of Rockefeller virus and a dose of human immune serum which must have been very close to the neutralizing dose. The time of onset of the paresis after inoculation and its general character make it almost certain that the monkey had a genuine infection with the Rockefeller virus attenuated by contact with immune serum. On subsequent testing it showed no resistance to the local virus.

Although the findings are compatible with the view that two immunologically distinct strains of virus are concerned, we feel that a more conservative interpretation is advisable unless it can be shown that monkeys *severely* paralysed by the Rockefeller virus are still susceptible to the Australian one. The work of Stewart and Rhoads (1929) has shown that there are definite degrees in the immunity that can be induced in monkeys by intradermal or subcutaneous inoculation of poliomyelitis virus. After a prolonged course of intradermal injections monkeys were found to be resistant to intracerebral inoculation of a moderately active virus (M.A.), but were not completely resistant to inoculation by the same route with a stronger virus (" Aycock "). Further, one monkey which had been proved resistant to " Aycock " was found still to be susceptible to the highly virulent " M.V.," which is the mixed Rockefeller strain used in our own experiments. Monkeys inoculated subcutaneously were less solidly immune. They resisted injections of " M.A.," but showed typical poliomyelitis after inoculation with " Aycock " instead of the abortive symptoms produced in the intradermally immunized animals. But all but one of the monkeys immunized by either method developed antibodies in their sera which were capable of neutralizing *in vitro* even the highly active " M.V."

In many respects these results are analogous to those reported above in showing the existence of degrees of immunity, but there is the important distinction that the monkeys used in our experiments had been frankly paralysed by the first attack of poliomyelitis. It has been the general opinion of experimenters that all monkeys which showed any definite poliomyelitic symptoms after inoculation, even a transient tremor, or ataxia were thereafter immune to inoculation with a virulent strain of virus. Flexner and Amoss (1924), for example, described an attenuated strain which rarely produced permanent paralysis, but which gave a solid immunity against an active virus.

It seems justifiable, therefore, to interpret our results as indicating definite minor immunological differences between the two strains used. The most rigid test of immunity is the intracerebral injection and, judged by this criterion, there is a relatively sharp difference between the two viruses. This is substantiated by the difference in neutralizing power for the two viruses shown by the serum of Monkey 64. On the other hand, there is definite evidence that the two are not completely distinct, since Monkey 64 was not further paralysed by the invariably fatal "M.V." virus, and since pooled human convalescent sera are approximately equally active against both strains (Burnet and Macnamara, 1929). It is of interest to note, however, that some recent tests with serum from Sydney convalescents has failed to show this parallelism, only the Victorian virus being neutralized. The number of experiments (two with each virus) is too small for the observation to be considered significant.

CONCLUSIONS.

A poliomyelitic virus derived from a child dying in Melbourne has shown distinct immunological differences from the Rockefeller Institute "mixed virus" strain both in cross-immunity experiments and by neutralization tests *in vitro*.

Three instances are described of monkeys which contracted a typical fatal infection after injection of the heterologous virus, despite the fact that some weeks previously they had suffered a typical attack of experimental poliomyelitis.

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A SPECIFIC PRECIPITATING POLYSACCHARIDE FROM *B. DYSENTERIÆ* (SHIGA).

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It has long been known that filtrates from bacterial cultures frequently contain a soluble product which gives specific precipitation with the homologous immune serum. The first attempt to isolate a specific precipitating substance was made by Pick (1902), who obtained from typhoid bacilli a substance which, while reacting specifically with homologous immune serum, failed to give any of the usual protein reactions. Fornet (1906) reported that the blood and urine of typhoid fever patients contained a highly reactive specific substance, and later Dochez and Avery (1917) isolated type-specific substances from the filtrates of actively growing pneumococcus cultures, and showed that these reactive substances were also present in the blood and urine of patients who were suffering from pneumonia. The later work of numerous investigators on specific substances, obtained from the filtrates and extracts of several bacterial species, has thrown considerable light on the chemical nature of these substances and the products of their hydrolysis.

The researches of Heidelberger and Avery (1923, 1925) on the specific polysaccharides which they obtained from the various types of the pneumococcus, when considered together with the observations of Griffith (1923, 1928) and Reimann (1926) on the virulence and cultural characters of these types, gradually led to the idea that the essential difference between a smooth and a rough culture in this species is related to the presence or absence of the corresponding polysaccharide in the antigen. The importance of this conception is far from being limited to the pneumococcus group, for the work of Zinsser and Parker (1923), Landsteiner and Furth (1927) and Bruce White (1927, 1929) indicates that a similar chemical difference exists in the smooth and rough variants of *B. typhosus* and the Salmonella group.

The separation of the smooth and rough variants of Shiga's bacillus by Arkwright (1921) was followed by investigations on the serological and chemical properties of these variants (White, 1929).

The present paper contains a preliminary account of the properties of a specific polysaccharide isolated from the smooth variant of Shiga's bacillus (Strain K 624). The culture used for the production of each batch of bacterial growth was derived from a carefully selected smooth colony on a plate.

The process of isolation first adopted consisted in concentrating the acid extract of the bacterial cells, and precipitating the specific substance from slightly alkaline solution by the addition of 2 volumes of alcohol. The preparation of a small quantity of the specific substance by this method

enabled the outstanding properties of the material to be studied, and the knowledge thus acquired suggested useful modifications in the methods of isolation.

TECHNIQUE OF ISOLATION.

The bacilli, after 36–48 hours' growth at 35° C. on a beef-infusion agar medium (pH 7·6), were washed off with distilled water in the proportion of one litre to 100 bottles. The area of the agar surface in each bottle was approximately 120 sq. cm. The dense bacterial suspension was made acid (pH 4) by the addition of acetic acid and heated at 100° C. for 15 to 20 minutes. Under these conditions the specific precipitating substance was set free from the cells and went into solution, the greater part of the bacterial protein being thrown down as a heavy white precipitate. The opalescent supernatant solution was then passed through a Berkefeld filter to clarify it and to remove the last traces of suspended bacterial protein. The clear filtrate was adjusted to pH 6 by the addition of caustic soda solution, and an excess of saturated normal lead acetate solution was added. A precipitate of insoluble lead-salts was obtained which contained only a very small amount of the specific precipitating substance. The addition of ammonia solution to the filtrate from the insoluble lead-salts brought down a large precipitate of basic lead-salts together with the whole of the active substance. The precipitate was removed by filtration, and after washing thoroughly with dilute ammonia solution, was re-suspended in about 500 c.c. of distilled water. The lead was removed in the form of the insoluble carbonate by passing a rapid current of carbon dioxide through the suspension heated at 60° C. The filtrate and washings obtained from the lead carbonate contained practically the whole of the specific precipitating substance. The addition of mercuric acetate solution to this filtrate, after thoroughly aërating to remove the excess of carbon dioxide, produced a further precipitate which contained very little of the active substance. The excess of mercuric acetate was removed by saturating the filtrate from the insoluble mercury salts with sulphuretted hydrogen and removing the mercuric sulphide by filtration. After thoroughly aërating the filtrate the volume of the solution was reduced to 30 or 40 c.c. by evaporation in a current of warm air. The solution was then filtered from any insoluble material, and the filtrate, after being made 0·1 N with hydrochloric acid, was dialysed against distilled water until it was free from the chloride ion. At this stage the solution was of a golden-yellow colour, and gave specific precipitation with the homologous immune serum when diluted hundreds of times with saline. After dialysis the solution was filtered, further concentrated to about 10 c.c., and heated on a water-bath with a small amount of Norit charcoal, which removed the greater part of the yellow colouring matter. The solution was then filtered and poured into eight times its volume of a mixture of equal parts of absolute alcohol and ether. The specific precipitating substance was thrown out of solution as a gummy mass, which, however, on re-dissolving in water and reprecipitating with absolute alcohol could be obtained as a white flocculent precipitate. The specific substance was further purified by fractional precipitation with increasing concentrations of alcohol. The precipitate first thrown down by

the addition of alcohol to the aqueous solution of the specific precipitating substance frequently contained a considerable amount of non-specific material.

The purification of the specific polysaccharide was followed at each step by means of the precipitin test with sera prepared by immunizing rabbits with a killed whole culture of the homologous organism. The yield of the specific substance obtained by the above methods from 100 bottles of bacterial growth varied between 200 and 500 mgm. in the different preparations.

TABLE I.

Batch No.	$[\alpha]_{5461}^{20°}$	Ash per cent.	Nitrogen per cent.	Per cent. reducing substance on acid hydrolysis (calculated as glucose).	Precipitation with immune serum.
12	37°	14·0	4·0	..	1/1,000,000
13	63°	..	..	..	1/4,000,000
16	70°	4·3	2·5	70	1/6,000,000
19	84°	4·2	3·9	52	1/6,000,000
20	58°	3·7	2·7	50	1/2,000,000*
21A	..	5·0	..	..	1/6,000,000
23	55°	2·2	3·7	55	1/3,000,000
23A	58°	2·2	2·9	..	1/4,000,000
29B	72°	2·0	2·5	66	1/6,000,000

Table I gives a summary of the properties of several batches of the specific precipitating substance. The product of batch 23 was further purified by re-dissolving in 0·1 N hydrochloric acid, dialysing until free from the chloride ion and re-precipitating with several volumes of acid alcohol. The resulting product 29B showed an increase in optical rotation and specific activity, whereas the nitrogen content fell from 3·7 per cent. to 2·5 per cent. Certain inorganic constituents are tenaciously retained by the specific polysaccharide in spite of repeated dialysis of the acid solution.

PROPERTIES OF THE SPECIFIC SUBSTANCE.

After several re-precipitations from alcohol the specific precipitating substance was obtained as a non-reducing, white amorphous powder, which gave a syrupy opalescent solution with small amounts of water and yielded a clear solution when further diluted. A 2 per cent. solution of the substance gave no precipitate when mixed with an equal volume of a saturated solution of the following reagents: tannic acid, uranium acetate, barium hydroxide, mercuric acetate, phospho-tungstic acid and normal lead acetate. An equal volume of a 30 per cent. solution of trichloracetic acid produced no precipitate. The biuret and ninhydrin tests on 10 mgm. of the specific substance were negative. The Molisch reaction was given by solutions of the substance to the limit of delicacy of the test, which with the reagents used was 1 part in 750,000. There was no colour reaction with iodine solution, and the Selivanoff reaction for ketoses was negative.

* The highest dilution tested.

All preparations of the specific polysaccharide obtained up to the present time have contained not less than 2·5 per cent. nitrogen, but at this stage it would be premature to decide whether nitrogen is a true part of the specific substance or not. On treating 32 mgm. of the polysaccharide for 30 minutes with nitrous acid in the micro-van Slyke apparatus no nitrogen was evolved. Analysis of the substance for carbon and hydrogen gave the following results: C, 43·9 per cent.; H, 6·9 per cent. The figures calculated for $(C_6H_{10}O_5)_n$ are C, 44·4 per cent., and H, 6·2 per cent.

TABLE II.

Time of heating (minutes).	Temperature, °C.	Per cent. reducing substances (calculated as glucose).	Limit of precipitation.
0	70	4·1	1/6,000,000
15	70	12·9	1/1,000,000
30	70	19·8	1/500,000
60	70	25·2	1/20,000
90	70	34·2	0
105	100	63·2	0
120	100	66·4	0

When boiled with an excess of N sulphuric acid the substance is hydrolysed with the production of about 65 per cent. of reducing sugars (calculated as glucose), but nothing is known as yet concerning the nature of the reducing substances liberated by acid hydrolysis except that the reducing solution does not give the usual tests for pentoses and glycuronic acid. Table II shows that quite early in the course of hydrolysis with N sulphuric acid the specific precipitating property of the polysaccharide with immune serum disappears.

PRECIPITATION WITH IMMUNE SERUM.

The tests were carried out by mixing 0·25 c.c. of the immune serum, diluted 1 in 5 with saline, with 0·25 c.c. of the dilution of the specific substance. The solutions were mixed, incubated at 35° for 4 hours and put into the cold room overnight. At a dilution of 1/6,000,000 the specific substance gave a definite precipitate. Control tests were frequently made using normal rabbit serum in place of the immune serum, but in no case was precipitation observed.

The serum obtained from a rabbit nine days after an intravenous immunization with increasing doses of the specific precipitating substance, amounting to 0·255 gm. in all, gave no specific antibody reactions.

A small amount of a polysaccharide has been isolated from the corresponding rough variant of Shiga's bacillus. The chemical and serological properties of this substance are left for discussion in a later paper. Work on the polysaccharide from the smooth variant described in this paper is being continued, and an attempt is being made to isolate specific substances from other strains of the dysentery group.

SUMMARY.

A method is described for the isolation of a polysaccharide from the smooth variant of *B. dysenteriae* (Shiga).

The substance obtained by this method gives specific precipitation with the homologous immune serum up to a dilution of 1 in 6,000,000.

The intravenous inoculation of this polysaccharide into a rabbit did not give rise to the production of demonstrable antibodies.

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THE RELATION OF THE NERVOUS SYSTEM TO A PATCHY ISCHÆMIA (BIER'S SPOTS) IN ANIMALS.

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THE effects of hæmorrhage and anhydræmia on the blood-supply to the tissues were recently investigated by Rous and his co-workers (Rous and Drury, 1929; Rous and Gilding, 1929, *a*), using a new method of vital injection. A peculiar distribution of the blood was observed; the circulation to the skin and certain internal organs was found to be irregular, some areas receiving blood, while others nearby were completely ischæmic and remained so for periods up to 1½ hours (Gilding and Rous, 1929). That this patchy ischæmia was not due to the lowered blood-pressure was demonstrated by reinjecting the defibrinated or heparinized blood, which was found to raise the pressure up to or more than that before the hæmorrhage without dispersing the ischæmia. The present work was undertaken to ascertain the exact part played by the nervous system in the production of this ischæmic patching. The phenomenon has been

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observed in rats, rabbits and cats, and is no doubt identical with "Bier's spots" in man. Cats were used throughout the following experiments.

The animals were shaved, or were depilated with barium sulphide, over the chest, abdomen and inner surfaces of the legs two or three days prior to the experiment. The cats were anæsthetized with ether and blood-pressures recorded from a carotid artery. About 25 c.c. of blood per kg. were taken from an axillary or carotid artery in two bleedings with a period of 20 minutes between. The dyestuffs—bromo-phenol blue or patent blue V—were made up with the appropriate amounts of NaOH and NaCl, so that the solutions were isotonic and of approximately pH 7.4: the amounts injected were 3 c.c. per kg. of a 4 per cent. solution of bromo-phenol blue and 1½ c.c. per kg. of a 6 per cent. solution of patent blue V.

The warmed dye solution was injected intravenously about 20 minutes after the last hæmorrhage. At the end of the experiments the carotid arteries were severed completely to exsanguinate the animals and an immediate *post-mortem* examination of the staining of the viscera made.

The first experiments were done on decapitated animals (with positive artificial respiration) in order to eliminate the activities of the vasomotor centre. A hæmorrhage of about 20 c.c. reduces the blood-pressure of spinal cats to about 30 mm. Hg.; further loss of blood incapacitates the heart. After a hæmorrhage of 20 c.c. the dye injection revealed ischæmic patching only in those areas where local pressures exert their influence, such as over the bony prominences, knees and xiphisternum (the animals were in the dorsal position and the limbs untied). The influence of local pressures on the cutaneous circulation was described by Rous and Drury (1929). The typical patching, which is to be seen over the whole skin of the intact animal, did not occur in the spinal animals. It seemed probable that the absence of true patching was due to the inadequacy of the bleeding, rather than to the absence of the vasomotor centre.

To test this, animals were bled, the dye injected, and a few of the ischæmic areas rapidly outlined with dots of indian ink; the animals were then decapitated and the marked areas observed thereafter. There was no immediate flooding of the unstained areas with dye as might be expected after the removal of vaso-constrictor impulses, but after some minutes the dye was observed to creep into the spots very slowly from the periphery, the centre of the patches remaining completely unstained. Occasionally, however, during an interval of 25 minutes after decapitation a feeble staining of the whole areas was observed. The experiment was varied by decapitating the bled animals before the dye injection. When this was done the patches were fewer in number and smaller than those to be seen in the intact animal, but nevertheless they did occur. Decapitation reduced the blood-pressure from an average of 85 mm. to about 30 mm. Hg. In four experiments varying amounts of defibrinated blood were injected intravenously, thus raising the blood-pressure to about 45 mm. Hg. This procedure had no effect on the dispersal of the ischæmic areas.

The *post-mortem* findings were as follows:

Spleen.—Evenly stained in most of the foregoing experiments, in marked contrast to the red spleens of normal depleted animals, in which only the Malpighian corpuscles receive the dye (Rous and Gilding, 1929, *a*).

Omentum.—Patchily stained in every instance.

Stomach and intestines.—Heavily and evenly stained. The bladder seldom showed the patching observed in intact bled animals.

Muscles.—In some instances evenly stained, in others the " mackerel sky " appearance, previously described (Rous and Gilding, 1929, *a*) was found. In general the pattern was found in animals exsanguinated soon after decapitation. This " mackerel sky " staining of muscles has been shown by Rous, Gilding and Smith (1930) to be due to differences in permeability of the capillary endothelium, the venous end of the capillary being most permeable, while the arterial end is least permeable.

The possibility that the spotting is produced by nervous influences and persists after their removal is not ruled out in the foregoing experiments. It was hoped that the spinal animal would settle this question, but as described above, the results of this procedure were indefinite.

In a further series of experiments the spinal cord was transected between the 9th and 10th thoracic vertebræ. Transection at this level has a negligible effect on the blood-pressure, hence it was possible to transect the cord before bleeding. The subsequent injection of the dye revealed typical patching of the skin above the lesion, whereas below, the ischæmic spots were smaller in size and fewer in number. Those below, however, were " true " spots. They were scattered over the skin of the abdomen and legs. In addition, as always, the skin over the bony projections was unstained. The abdominal viscera were as in bled intact animals. The muscles above the lesion showed the pronounced pattern, but below, the muscles were invariably evenly stained. The even staining of muscles after section of sciatic and femoral nerves was described and discussed in previous papers (Rous and Gilding 1929, *a* ; Rous, Gilding and Smith, 1930).

These experiments clearly demonstrate that patching of the skin occurs in the absence of control by the principal vasomotor centre, but do not exclude the possibility that spinal centres could still function. This latter factor was ruled out by pithing the cord below the transection before bleeding the animals. The results obtained after this procedure were identical with the foregoing experiments, except that the stained skin between the ischæmic patches contained more dye than did the stained skin above the transection. In one of these experiments the pads of both hind feet were patched (in normal bled animals the pads are invariably well stained).

The diminution in size and number of the ischæmic patches after removal of nervous influences is no doubt due to the removal of the vaso-constrictor tone of neighbouring vessels in which the circulation continues. This allows greater blood-flow through the already stained areas and consequently greater diffusion of dye from them to the adjacent unstained skin. Confirmation of this view is found in the experiments in which the spinal cord was pithed, where, as noted above, the stained areas in the denervated skin were more heavily stained than were similar areas in the skin above the lesion. Incidentally, this indicates a vaso-constrictor tone of spinal origin, for the heavy staining of skin was never seen in any of the simple transection experiments.

The close similarity of the ischæmic patching in animals to " Bier's spots " in man was discussed by Rous and Gilding (1929, *b*). " Bier's spots " are

known to occur in completely denervated skin (Bier, 1898; Wolf, 1924). The demonstration that the ischæmic spots in animals are not the direct result of nervous influences strengthens the assumption that Bier's spots in man, and ischæmic patching in animals, are one and the same phenomenon. The part played by the nervous system in this phenomenon may be summarized as follows: The needs of the organism bring about a vaso-constriction, more pronounced in some areas than in others. The vaso-constriction diminishes the blood-flow, and this is probably the primary cause of the patching. In diseases such as Raynaud's and thrombo-angiitis obliterans similar facts emerge. The blood-flow is diminished, in one case by cold, and in the other by arterial occlusion; "Bier's spotting" follows, and if the local blood-flow is not increased, gangrene sets in.

The above experiments shed no light on the ultimate mechanism by which this intense constriction of the peripheral vessels is produced.

SUMMARY.

1. The patchy ischæmia observed after hæmorrhage occurs in the absence of vasomotor control of the minute vessels concerned.

2. The compensatory vaso-constriction which occurs after hæmorrhage is a predisposing cause of the intense local ischæmia observed.

3. The production of ischæmic patching in animals in denervated areas is in agreement with observations on Bier's spots in man.

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CATAPHORESIS EXPERIMENTS WITH TYPHUS VIRUS.

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THE nature of the virus of typhus fever still remains obscure. This virus is often classed among the ultra viruses (Rivers, 1928), although the filtration experiments by Rickets and Wilder (1910), Anderson and Goldberger

(1912) and Olitsky (1917), as well as the more recent ones by Zinsser and Batchelder (1930), seem to establish that, in so far as filterability is concerned, it differs from the group of ultra- or filterable viruses. Furthermore, Mooser's work on Mexican typhus (1928, 1929), confirmed by Maxcy (1929) and by Zinsser and Batchelder (1930), supports the ætiological relationship to *rickettsia*.

In the course of studies on the virus of European typhus, we have conducted a series of cataphoresis experiments in order to compare its behaviour in this respect with that of filterable viruses. The object of this paper is to present results of these experiments. These results indicate another point of difference between the typhus virus and certain filterable viruses.

TECHNIQUE.

In all of our experiments, except where otherwise stated, brain material from infected guinea-pigs, taken on the fourth or fifth day of fever, was used. The cataphoresis was carried out in the U-type of apparatus described by Todd (1927), as well as in the one described by Kligler (1931), with identical results. The technique consisted in emulsifying a given portion of the brain of an infected guinea-pig in 0·5 per cent. saline solution, centrifugalizing slowly to remove larger particles and diluting the supernatant fluid, 1 part in 10, with M/50 solution of the required buffer mixtures. The current was passed through for 2 to 3 hours, and the material from the middle vessel as well as from the respective poles tested for virus by animal inoculation. From time to time the virus was tested by inoculation into rabbits and subsequent agglutination of the serum with OX, 19.

The experiments may be divided into three groups:

1. Designed to determine the charge of the virus and the direction of flow in solutions of different hydrogen-ion concentration.
2. Attempts to recover virus by cataphoresis of suspensions of brain from animals which had recovered from an infection.
3. Separation of virus from a virus-antibody mixture by cataphoresis.

EXPERIMENTAL.

The Electric Charge of Typhus Virus.

Experiment 1.—Half a brain from an infected guinea-pig, killed on the fourteenth day after the infection and sixth day of fever, was emulsified in 8 c.c. distilled water. The suspension was centrifugalized for 5 minutes at 1000 revolutions, and 2·0 c.c. of the supernatant fluid diluted with 20 c.c. M/45 solution of a mixture of phosphate buffers of pH 7·0. Our apparatus was used and the current allowed to run for 2 hours. At the end of this period there was no appreciable warming of the side arms; the tube at the positive pole was markedly turbid, while that at the negative pole was clear. Material withdrawn from each pole was injected into a guinea-pig. The guinea-pig inoculated with material from the positive pole went through a typical infection, while the one which received material from the negative pole remained normal. On subsequent re-infection with virus the former remained unaffected, while the latter developed a typical infection.

Experiment 2.—The procedure in this experiment was essentially the same as in the previous one, except that simultaneous tests were carried out at pH 6·0, 7·0 and 8·0 respectively. The duration of this experiment was only 1 hour. The results are given in Table I:

TABLE I.

pH.	Pole.	Amount injected.	Condition of fluid at end of cataphoresis.	Result of infection.	Result of reinfection.
8	+	4·5 c.c.	Turbid up to top of side arm	+	—
8	—	4·0 c.c.	Clear	—	+
7	+	3·0 c.c.	Moderate turbidity ⅓ of tube	+	—
7	—	3·5 c.c.	Clear	—	+
6	+	2·5 c.c.	Slight turbidity ½ of tube.	+	—
6	—	3·0 c.c.	Clear	—	+

Experiment 3.—Half a brain of guinea-pig 101, removed on the third day of fever, was triturated in 10 c.c. of 0·5 per cent. saline solution, and centrifugalized three minutes at 1000 revolutions. The supernatant fluid was diluted 1 to 10 in citrate-phosphate buffer mixtures of different pH, diluted 1 part in 9 parts 0·5 per cent. saline solution. The apparatus designed by us was used. The cataphoresis lasted 3 hours.

The results are shown in Table II:

TABLE II.

pH.	Pole.	Condition of fluid at end of cataphoresis.	Result of injection.
6·0	+	Definitely turbid entire length of tube	+
	—	Clear	—
	Middle flask	..	..
5·0	+	Slight turbidity, lower part of tube	—
	—	Clear	—
	Middle flask	Flocculation	—
4·0	+	Clear	—
	—	,,	—
	Middle flask	Heavy precipitate	—

These experiments as well as others of the same character showed that between pH 6·0 and 8·6 the typhus virus carried a negative charge. In solutions of pH 4·0 and 5·0 there is flocculation and the virus is apparently destroyed during the period of cataphoresis, 2–3 hours. Table III presents the results of one experiment giving more detailed data concerning the course of infection.

TABLE III.

	Infection.				Reinfection.		
pH.	Pole.	Incubation, days.	Duration of fever, days.	Maximum temperature, °C.	Incubation, days.	Duration of fever, days.	Maximum temperature, °C.
8·0	+	7	9	41·0	—	—	—
8·0	—	—	—	—	6	9	40·6
7·0	+	9	9	41·4	—	—	—
7·0	—	—	—	—	6	10	41·3
6·0	+	7	9	41·0	—	—	—
6·0	—	—	—	—	5	10	41·1

Recovery of Virus from Brains of Animals which have Recovered from an Infection.

We now attempted to ascertain whether it is possible by cataphoresis to recover typhus virus from the tissues of animals which had recovered from an infection, as Olitsky and Long (1929) have done from tissues of rabbits which had recovered from vaccinia. Brain material both from rabbits and guinea-pigs as well as tunica and testis from guinea-pigs were used. Alternate and parallel tests were made with the Todd apparatus as well as our own. Citrate-phosphate buffer mixtures of pH 6·5 were used, and the current was run through 2 to 3 hours. Only the turbid material from the positive pole was injected. As a rule the animals were re-injected 3 to 4 weeks later to test for immunity.

Eight experiments involving 25 animals were made. In 8 animals brain suspensions and in 17, suspensions of both brain and tunica-testis were subjected to cataphoresis. The animals were killed 7 to 18 weeks after the infection.

The results were uniformly negative. In no case did we obtain any effect suggestive of an infection. It seems that in the case of typhus virus it is not possible by cataphoresis to demonstrate the presence of virus in the tissues of animals which had recovered from an infection.

Recovery of Typhus Virus from Mixtures of Virus and Immune Serum.

In view of the failure to recover virus from immune animals, it seemed of interest to ascertain whether virus could be recovered from virus-containing materials mixed with immune serum. The procedure employed was briefly as follows :

Blood withdrawn at the height of fever was defibrinated and diluted with 2–3 volumes of a M/45 phosphate buffer solution having a pH 7·0. The brain emulsions were prepared as described above and diluted to 10 times with the same buffer solution. Given portions of the diluted virus material were then mixed with serum from recovered animals and subjected to cataphoresis. Parallel tests were made with virus mixed with normal guinea-pig serum.

Experiment 4.—Guinea-pig 1 was bled 30 days after the infection or about 15 days after the cessation of fever, and the serum used for neutralization. The virus was taken from guinea-pig 3 on the fourth day of fever. The defibrinated blood was diluted with 2½ volumes and the brain suspension with 9

volumes of buffer solution. The diluted materials were then thoroughly mixed with immune serum in the proportion indicated below and subjected to cataphoresis. The reaction of the buffer solution was pH 7·0 and the duration of the cotaphoresis 1½ hours. The specificity of the virus was established by injection into a rabbit. The results of the animal inoculation are given in Table IV :

TABLE IV.

Virus suspension in c.c.	Serum.		Results of infection with material from—			Reinfection of animals which received—		
	Normal in c.c.	Immune in c.c.	+pole.	−pole.	serum-virus mixture.	+pole.	−pole.	mixture.
Blood 15·0	3·0	0·0	+	−	+	−	+	−
,, 15·0	0·0	3·0	−	−	±*	+	+	−
Brain 10·0	3·0	0·0	+	−	+	−	atypical fever	−
,, 10·0	0·0	3·0	−	−	+	±†	+	−

(*) Aborted infection, 3 days fever. (†) Aborted infection, 1 day fever.

Experiment 5.—Immune serum obtained from guinea-pig 5 bled 26 days after infection. The virus was obtained from guinea-pig 6 on the fifth day of fever. The preparation was the same as in Experiment 4. The reaction of the buffer was pH 8·6, and the duration of cataphoresis was 2 hours. The positive poles became turbid, the negative remained clear. The results are tabulated (Table V) :

TABLE V.

Virus suspension in c.c.	Serum.		Results of infection with material from—			Reinfection of animals which received—		
	Normal in c.c.	Immune in c.c.	+pole.	−pole.	serum-virus mixture.	+pole.	−pole.	mixture.
Blood 20·0	2·0	0·0	+	−	+	−	not tested.	
,, 20·0	0·0	2·0	+	−	+	not tested.		
Brain 10·0	2·0	0·0	+	−	+	−	+	not tested.
,, 10·0	0·0	2·0	+	−	+	−	+	−

In the first experiment there was apparently only partial neutralization, but the reaction was more complete at the positive pole than in the mixture in the middle vessel. The results indicate that both virus and antibody wander in the same direction, and that a separation cannot be effected. In the second experiment the serum was evidently of little potency, having been withdrawn too soon after recovery from the infection.

The experiments were repeated, using pooled serum from several recovered animals.

Experiment 6.—Sera from three guinea-pigs taken 12 days after recovery were pooled. Virus was taken from guinea-pig 22 on the fourth day of fever.

The procedure was the same as above except that phosphate buffer at pH 6·0 was used and the duration of the cataphoresis was 1½ hours. The negative poles remained clear; the positive poles were turbid for a distance of 4–5 cm. About 2 c.c. from each pole and from the middle vessel respectively were injected into test guinea-pigs (Table VI).

TABLE VI.

Virus suspension in c.c.	Serum. Normal in c.c.	Serum. Immune in c.c.	Results of infection. +pole.	Results of infection. −pole.	Results of infection. central vessel.	Results of reinfection. +pole.	Results of reinfection. −pole.	Results of reinfection. central vessel.
Blood 20·0	2·5	0·0	+	—	+	..	+	..
,, 20·0	0·0	3·0	±	—	+	—	inter-current infection	..
Brain 10·0	2·5	0·0	+	—	+	—	+	—
,, 10·0	0·0	3·0	—	—	+	—	atypical fever	—

Experiment 7.—Pooled immune serum from three guinea-pigs taken 12 to 14 days after recovery was used. The virus was taken on the sixth day of fever. The preparation was the same as in Experiment 6, except that the buffer solution was pH 8·6 and the duration of the cataphoresis was 2 hours (Table VII).

TABLE VII.

Virus suspension in c.c.	Serum. Normal in c.c.	Serum. Immune in c.c.	Results of infection. +pole.	Results of infection. −pole.	Results of infection. central vessel.	Results of reinfection. +pole.	Results of reinfection. −pole.	Results of reinfection. central vessel.
Blood 20·0	3·0	0·0	+	—	+	—	..	—
,, 20·0	0·0	3·0	—	—	+	—	?	—
Brain 20·0	3·0	0·0	+	—	+	..	+	..
,, 20·0	0·0	3·0	±	—	—	—	..	—

In both of these experiments the neutralization was incomplete. It would seem that either the ratio of virus : immune serum or the length of time they were in contact was not adequate. It is clear, however, from these experiments that it is not possible at the reactions used (pH 6·0 and 8·6 respectively) to obtain a separation of virus from antibody.

SUMMARY AND CONCLUSION.

Experiments are reported bearing on the electric charge of typhus virus and the possibility of recovery of virus by cataphoresis from tissues of immune animals. The results of the experiments show:

1. That the typhus virus wanders to the positive pole. This may signify either that the virus carries a negative charge or that it is carried by the associated proteins.

2. It has not been possible by cataphoresis to obtain virus from tissues of animals which had recovered from a typhus infection.

3. It has not been possible to separate virus from virus-antiserum mixtures by cataphoresis of suspensions at pH 6·0 and 8·6 respectively.

4. In so far as recovery of virus from tissues of immune animals is concerned, the typhus virus differs from the filterable viruses studied by Olitsky and his associates.

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A METHOD OF DRYING COMPLEMENT FROM THE FROZEN STATE.

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FOR a number of years dried guinea-pig complement has been prepared and used in this laboratory. The method employed at first was that described by Hartley, Eagleton and Okell (1923), in which the complement is dehydrated by distilling off the water *in vacuo*. This treatment involves constant foaming of the complement and the dried product shows in consequence some loss of activity. In order to counteract this loss the practice of adding sodium chloride to the complement before drying was adopted (Tulloch, 1928).

The present method takes advantage of the following facts:

(1) Water may be frozen if rapid evaporation be brought about under suitable conditions.

(2) Water may pass from the solid to the gaseous state without obviously passing through the aqueous phase.

(3) Complement retains its activity best at a low temperature. The apparatus required is simple, only a vacuum pump (Geryk) and a desiccator of suitable design being necessary. The desiccator (Fig. 1) contains dry calcium chloride, a thin glass dish to hold the complement and a support for the latter.

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The support should be so designed that it virtually brings about thermal isolation of the complement and at the same time occludes as little space as possible. An X-shaped wire frame, the four ends of which are fixed into pieces of cork, forms a suitable support. The dimensions of the dish depend on the volume of complement to be dried, and should be such that the depth of fluid is at least one centimetre. There should be ample clearance between this dish and the sides of the desiccator and the calcium chloride.

The guinea-pig serum is placed in this dish and the desiccator is luted down with rubber-vaseline compound.* The desiccator is now evacuated. Slight bubbling will take place, and the temperature of the complement will drop rapidly until it suddenly freezes solid. Previous chilling of the complement will hasten freezing, but this is unnecessary, for freezing will occur in any case in approximately 10 minutes. When the complement has become frozen the desiccator tap is closed and the apparatus is left at room temperature. No further supervision is necessary for the complement will remain frozen until dry because the evaporation of the water automatically keeps it several degrees below 0° C. Drying of the complement under such conditions is surprisingly rapid; six hours suffice to dry 50 c.c. of complement, but it is advisable to leave it drying overnight. The removal of the dried complement presents no difficulty, for it spontaneously detaches itself from the glass surface in the form of pink, readily pulverizable masses. Any dry serum forming a thin film firmly adherent to the glass should be discarded. The dried complement should be stored *in vacuo* over calcium chloride at a low temperature. The ratio of dry weight to original fluid volume will, of course, have been determined in order to ascertain the volume of distilled water required to reconstitute the complement from a given weight of the dried product. On an average 1 gm. of dried complement is equivalent to 16 c.c. of original complement. The dried complement dissolves in the required volume of distilled water very rapidly but it is advisable to allow the solution to stand for at least half an hour before ascertaining its hæmolytic titre.

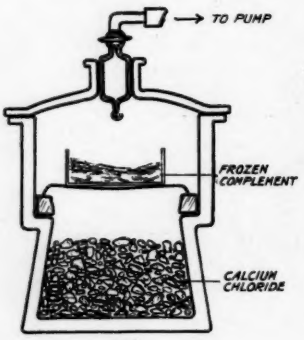

FIG. 1.

This method has the following advantages over the method of vacuum distillation of the water at 37° C.:

(1) The complement is automatically kept below 0° C. until it is perfectly dry.

(2) The frozen complement cannot froth, and therefore the globulin fraction is not exposed to the denaturing influence of an extensive air-water interface.

(3) Complement reconstituted by addition of distilled water to the dried product is indistinguishable in appearance and hæmolytic titre from the original.

* Rubber solution (chemico) 1 part; vaseline 2 to 5 parts; heat until all solvent has evaporated.

(4) Once the complement becomes frozen the drying process requires no supervision, and thus a dried product may be obtained with only a fraction of the labour required for the method previously used.

One pound of dry calcium chloride will dry 75 c.c. of complement and leave a 50 per cent. margin of safety. When employed for the Wassermann reaction, dried complement has the advantage of providing a standard product which represents the pooled serum of a number of guinea-pigs, which permits of tests being carried out on many different dates with the same batch and which reduces wastage to a minimum. In conclusion, it might be pointed out that the method described may be applied to obtain dry, stable preparations of other biological products and of certain infective agents.

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TETANUS.—X: THE EFFECT OF THE OXYGEN TENSION OF THE TISSUE FLUIDS IN CONTROLLING INFECTION BY *B. TETANI*.

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IN previous papers one of us (Fildes, 1927, 1929, *a*, 1929, *b*) has advanced the view that the inability of tetanus spores to germinate in the normal animal is due to the oxygen tension in the tissue fluids poising the Eh of these fluids at a level positive to that at which tetanus spores can germinate. In the present paper is shown the effect of artificial variation of the oxygen tension in the tissue fluids upon germination of the spores.

PROCEDURE FOR ALTERING THE OXYGEN TENSION IN THE TISSUES.

The oxygen tensions in the tissue fluids were lowered by altering the composition of the air breathed, the animals being enclosed in two air-tight chambers (capacity about 670 litres), and provided with sufficient food, water and bedding for four or five days. These chambers, which are described elsewhere (Campbell, 1927, *a*, *b*), were fitted with fans to keep the air constantly mixed. The carbon dioxide produced by the animals was absorbed by soda lime and the oxygen used was automatically replaced.

* Working under grants from the Medical Research Council.

In some experiments carbon monoxide was added to the air in gradually increasing quantities until the concentration was about 0·15 per cent.; this level was reached sometimes in five days and sometimes in fourteen days. In this way the oxygen tension in the subcutaneous region of a guinea-pig was reduced from the normal figure of about 20 mm. Hg. to about 6 mm. Hg. (Table I), and that in the abdominal cavity from about 29 to about 8 mm. Hg., *i. e.* the tissue oxygen tensions were lowered by about 70 per cent. The animals lost appetite and weight but usually all survived the experiments, apart from those killed by tetanus. The tensions were estimated by the method of injection (Campbell, 1924) of a small amount of nitrogen into the abdominal cavity and under the skin; samples of this gas were analysed after sufficient time had elapsed to allow constancy to become established. The tensions obtained are regarded as those existing in the tissue fluids, *e. g.* serous, lymph, with which the gas is in contact; these tensions are probably the same inside the cells of the tissues concerned.

TABLE I.—*Tissue Oxygen Tensions of Guinea-pigs under Various Conditions.*

Condition.	Number of guinea-pigs.	Subcutaneous oxygen tensions.			Oxygen tensions in abdominal cavity.		
		Limits (mm. Hg.)	Average (mm. Hg.).	Percentage of normal.	Limits (mm. Hg.)	Average (mm Hg.).	Percentage of normal.
Normal . .	5	18–23	20	100	27–32	29	100
Breathing 0·15% CO	5	5–8	6	30	7–10	8	27
,, 7% O_2	6	2–8	5	25	5–11	8	27
,, 60% O_2 .	6	25–28	26	130	36–39	38	130

The fall of oxygen tension in the tissues produced by carbon monoxide was due to anoxæmia, the poison combining with hæmoglobin and reducing its oxygen capacity. At the same time the breathing was increased and carbon dioxide was removed from the blood in greater quantity than normal, so that the carbon dioxide tensions in the tissues were decreased from about 47 to 37 mm. Hg. and from 49 to 43 mm. Hg. in the subcutaneous region and abdominal cavity respectively (Table II). These changes for guinea-pig's tissue tensions under carbon monoxide poisoning resemble very closely those previously obtained with rabbits (Campbell, 1929).

TABLE II.—*Tissue Carbon Dioxide Tensions under Various Conditions.*

Condition.	Number of guinea-pigs.	Subcutaneous carbon dioxide tensions.			Carbon dioxide tensions in abdominal cavity.		
		Limits (mm. Hg.)	Average (mm. Hg.).	Percentage of normal.	Limits (mm. Hg.)	Average (mm. Hg.).	Percentage. of normal.
Normal . .	5	42–57	47	100	46–53	49	100
Breathing 0·15% CO	5	28–41	37	78	38–46	43	87
,, 7% O_2 .	6	28–33	30	64	23–32	27	55
,, 60% O_2	6	51–54	52	110	49–60	55	112

In other experiments the oxygen tensions in the tissues were lowered by enclosing the animals in the same chambers as above with gas containing oxygen at pressures gradually decreasing from the normal, *i. e.* 20·9 per cent. to

about 7 per cent., three or four days being taken to reach this level. In this way the animals, apart from those killed by tetanus, survived the experiments, which lasted only six or seven days. The effects upon the tissue oxygen tensions (Table I) were very similar to those produced by carbon monoxide—that is, they were lowered by about 70 per cent. The carbon dioxide tensions were also decreased (Table II), and to a greater extent than with carbon monoxide.

Both with carbon monoxide and with low oxygen pressure in the gas breathed the results on tissue gas tensions for different animals were regular, showing only slight variations, as will be seen from the limits given in the tables.

In order to increase the oxygen tensions in the tissues the oxygen pressure in the gas in the respiratory chamber was increased from the normal 20·9 per cent. to 60 per cent., at which pressure oxygen may be breathed for long periods without risk of pneumonia. The effects upon tissue gas tensions are shown in Tables I and II. The oxygen tension in the abdominal cavity was constantly increased, the average increase being 30 per cent., or 9 mm. Hg., *i. e.* from 29 to 38 mm. Hg. Sometimes much higher increases were observed in guinea-pigs and in other animals (Campbell, 1927, *a*, *b*). The oxygen tensions in the subcutaneous region under high oxygen pressure were usually increased, sometimes very markedly, *e. g.* by 50 per cent. and more, but at other times irregularities were observed which were considered to be due to constriction and relaxation of vessels. These irregularities were not observed in the abdominal cavity. The figures given in the tables show the results for tissue tensions during the first few days of exposure to high oxygen pressure, this period covering the time of germination of the spores. The carbon dioxide tensions in the tissues were always increased, on an average by about 10 per cent. (Table II), by high oxygen pressure in the air.

RESULTS OF THE INOCULATION OF TETANUS SPORES.

The tetanus spores used in all experiments were obtained from surface cultures of a toxic strain, washed and heated to 65° C. for 1½ hours. They were suspended in distilled water in a concentration of about 160 million spores per c.c. The same suspension was used throughout.

Spores alone in Guinea-pigs with Reduced Oxygen Tension.

A few preliminary experiments were carried out, in one of which 7 guinea-pigs were inoculated with 0·5 c.c. (*i. e.* 80 million) tetanus spores, and exposed to carbon monoxide, so that their tissue oxygen tensions were lowered by about 70 per cent. as in Table I. Tetanus did not develop.

Spores and Aleuronate Injected Subcutaneously in Guinea-pigs with Reduced Oxygen Tension.

In these and in all subsequent experiments 0·1 c.c. of the spore suspension, diluted 1/100, mixed with 0·2 c.c. aleuronate was injected—*i. e.*, approximately 160,000 spores. The aleuronate was the usual autoclaved mixture of 3 per cent. corn starch and 3 per cent. aleuronate in broth. The inoculum was injected subcutaneously over the chest-wall.

In this series 61 per cent., or 8 out of 13 guinea-pigs exposed to carbon monoxide, died, while only 37 per cent., or 7 out of 19 control animals, died. Owing, however, to the absence of local tetanus in animals so inoculated and the fact that most of them died during the night, it was not possible to be certain that death in all cases was due to tetanus.

Spores and Aleuronate injected Intramuscularly in Guinea-pigs with Low and High Oxygen Tensions.

In these experiments the animals were inoculated with the same mixture intramuscularly in the right thigh. In animals so inoculated the occurrence of local tetanus is obvious and could be observed through the large glass window of the chamber.

TABLE III.—*Summary of Results from Intramuscular Inoculations.*

Experiment No.	Conditions.	Tetanus positive.		
		High oxygen pressure.	Normal oxygen pressure, i. e. controls.	Low oxygen pressure.
1	CO	..	30% (3 out of 10)	75% (6 out of 8)
2	CO	..	44% (7 ,, 16)	75% (27 ,, 36)
3	CO	..	55% (21 ,, 38)	75% (15 ,, 20)
4	High O_2 (60%) and low O_2 (7%)	6% (1 out of 17)	30% (5 ,, 17)	82% (14 ,, 17)
5	High O_2 (60%) and low O_2 (7%)	Ditto	47% (8 ,, 17)	70% (12 ,, 17)
	Totals	6% (2 out of 34)	45% (44 out of 98)	75% (74 out of 98)

In the five experiments illustrated in Table III the mixture of aleuronate and tetanus spores was injected into the right thigh muscles and tetanus developed three or four days later in about 45 per cent. of the control animals—that is, the animals breathing pure air. In three experiments (Nos. 1, 2 and 3) the animals were exposed to carbon monoxide so that their tissue oxygen tensions were lowered to about 6 mm. Hg.; tetanus developed in about 75 per cent. of these animals. In each of the two remaining experiments 51 animals were employed, divided into 3 batches of 17 each, one batch (control) being exposed to pure air, the second batch being enclosed in a chamber with 7 per cent. oxygen, and the third batch being enclosed in another chamber with 60 per cent. oxygen. In all cases the barometric pressure was the same as that of the outside air, viz. about 750 mm. Hg., the chambers being filled with the proper mixture of gas to give the required composition. Air temperature was kept at about 20–25° C. In Table III the carbon monoxide experiments are classed with the low oxygen pressure experiments because, as already stated, the effects upon tissue oxygen tensions were similar. Under high oxygen pressure only 6 per cent. of the inoculations produced tetanus, whilst under low oxygen pressure the figure was 75, that for the controls being 45. It is obvious that as the oxygen pressure in the air breathed decreased the number of tetanic animals increased and *vice versâ*. Thus, by altering the oxygen pressure in the air breathed it is possible to control to a great extent the number of animals developing tetanus.

DISCUSSION.

The results are in agreement with the view of the effect of oxygen tension already referred to. When the tissue oxygen tensions in the abdominal cavity were 8, 29 and 38 mm. Hg., the percentages of " takes " were 75, 45 and 6 respectively.

The measurements of tension given in Table I were not obtained from the animals actually inoculated, but there is no reason to doubt that similar tensions would be found in any guinea-pigs. Neither was it possible to measure the tensions in the muscles into which the inoculations were made, but there is a general resemblance in tissue tensions in most regions of the body (Campbell, 1931). According to the view upon which these experiments were based the action of the aleuronate is to supply a minute circumscribed mass of phagocytes and spores in which the oxygen tension will be lower than the normal. The tension in such a mass will be the normal at the periphery and progressively lower towards the centre. If a spore happens to be found in a zone in which the tension is sufficiently reduced, it will germinate and produce toxin. By adjustment of the constituents of the inoculum, these conditions are found in some 45 per cent. of the control animals (Table III). If, however, the oxygen tension of the surrounding fluids is reduced artificially, the general level of the tension gradient in the mass will be lower and the conditions of low oxygen tension are more likely to be fulfilled. Conversely, if the tension is raised the conditions are less likely to be fulfilled. In the former case more animals become tetanic, in the latter fewer.

CONCLUSIONS.

When guinea-pigs are inoculated with mixtures of tetanus spores and aleuronate, the incidence of germination and tetanus can be reduced by increasing the oxygen tension of the tissue fluids. Conversely, by reducing the oxygen tension the infection is facilitated.

These results support the view previously advanced that the oxygen tension of the tissue fluids controls the germination of tetanus spores.

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A NOTE ON THE EXPERIMENTAL TRANSMISSION OF "LOUPING-ILL" TO MICE.

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ENDEAVOURS to transmit the louping-ill infection to rabbits, guinea-pigs, pigeons, mice and a monkey by means of various tissues and body-fluids of sheep and by ticks infesting them have been reported as unsuccessful (Meek and Greig-Smith, 1896; Departmental Committee Report, 1906; Stockman, 1916, 1918). More recently, Pool, Brownlee and Wilson (1930) have been able to reproduce the condition in sheep and pigs by intracerebral inoculation of an emulsion of brain or spinal cord from diseased animals. This work was done at the Moredun Institute of the Animal Diseases Research Association, Edinburgh, and in continuation of it, a Berkefeld filtrate* from an emulsion of the brain of an experimentally infected animal when inoculated intracerebrally in sheep was also stated to be capable of reproducing the characteristic disease. The infective tissue and filtrate contained no demonstrable organisms, and the disease could also be reproduced in passage experiments in which sterile filtrates were used for injection. These data indicated that the pathogenic agent was a filtrable virus, and it seemed possible that it might have affinities with the known neurotropic viruses. Attempts were then made to transmit the infection to various laboratory animals with a view to facilitating, if possible, the laboratory study of both the virus and the experimental disease. No disease was observed after intracerebral inoculation of rabbits, guinea-pigs and rats with either filtered or unfiltered suspensions of brain capable of infecting sheep. In the case of rabbits, young animals were chosen and the intra-ocular or the vertical route, or both, used in different cases, but, as with the other animals, no symptoms of infection resulted during long periods of observation. In mice, however, definite symptoms were produced and we believe experimental "louping-ill" may be readily established in these animals.

TECHNIQUE.

Sheep brain was ground up in a sterile mortar in presence of the smallest effective quantity of fine sand. The volume of brain was then measured as accurately as possible, and thoroughly emulsified in sterile saline or sterile broth to make a 10 per cent. suspension. Various methods were tried to clarify this fluid prior to filtration. Silk and filter-paper proved ineffective, and ultimately centrifugalization was used with satisfactory results. The speed

* Prepared by Prof. Mackie from brain-tissue submitted by Dr. Russell Greig for filtration tests.

was the lowest possible on the available centrifuge (600 r.p.m.) and the time did not exceed 7 minutes. In this way a product was obtained which allowed sufficient fluid to pass the filter candle.

Filtration was through a British Berkefeld candle. A manometer was used on the exhaust system and the negative pressure did not exceed 620 mm. of mercury. The time taken was always under 15 minutes, and filtration was carried out at room temperature.

The usual aërobic and anaërobic sterility tests were employed, and in every case the filtrate showed complete absence of bacteria by ordinary cultural methods.

Inoculation of mice was by the intracerebral route, the skin of the scalp being depilated and cleansed under slight ether anæsthesia. The animal was completely anæsthetized for the inoculation. A fine-bore needle attached to a 1 c.c. syringe (as used for intradermal tests) was employed, and it was found to penetrate the skull without difficulty. The site of inoculation was just posterior and lateral to the vertex, and the point of the needle was carried through the skull for a distance of $\frac{1}{8}$ to $\frac{1}{4}$ in. The volume of filtrate introduced was approximately 0·05 c.c., but the quantity was largely governed by the condition of the animal. With increase of intracranial pressure the respiration, from being rapid and regular, changed to a slow irregular rhythm, and this change was taken as the indication to withdraw the needle.

THE EFFECT OF THE INOCULATION OF SHEEP-VIRUS IN MICE.

In all, 7 sheep brains representative of three separate field strains of the infective agent have been used for mouse inoculation, and in each case symptoms very similar to the disease as it occurs in sheep have been produced. The field strains had been preserved for periods up to 7 months in glycerol at low temperature before being injected into the sheep which supplied the brain tissue used in our experiments. No differences were found among the three strains as regards degree of infectivity, pathogenic effects or incubation period.

Field Virus No. 1 was represented by 5 sheep brains. Of 16 mice which were injected intracerebrally with filtrates from these brains, 13 showed typical and fatal infection with the symptoms described below, and 2 died within 3 days after injection without showing any evidence of infection. The remaining mouse showed modified symptoms after 17 days; apparent recovery occurred, but 6 weeks after inoculation a typical and fatal illness arose. Table I shows the record of mice injected with filtered emulsion from one of the sheep brains of Field Strain 1. In addition, the table shows the passage of the infection to more mice and the contact infection of uninoculated mice. These findings are described below. The other two field strains were each inoculated into 2 mice after filtration and typical infections occurred. A second passage of the infection was procured in each case.

The *incubation period* before symptoms appeared was relatively constant when filtrate of fresh sheep brain was used as inoculum. In 13 mice so inoculated the time of appearance of the first symptom referable to the central nervous system was from 5 to 9 days. In some cases prodromal symptoms preceded the main symptoms by 12–24 hours. Such *prodromata* included

TABLE I.—*Record of Infections in Mice Resulting from Filtrate of Emulsion of Sheep Brain No. 925.*

Filtrate prepared 18 . 6 . 30.

Mouse No. 1:
18 . 6. I.C. injection.
25 . 6. Typical symptoms. Died.
26 . 6. Brain removed and filtered.

Mouse No. 2:
18 . 6. I.C. injection.
24 . 6. Marked symptoms. Killed.
Brain removed and filtered.

Mouse No. 6:
26 . 6. I.C. injection.
4 . 7. Typical symptoms.
5 . 7. Died.

Mouse No. 7:
26 . 6. I.C. injection.
2 . 7. Typical symptoms. Died.
Brain removed and filtered.

Mouse No. 13:
24 . 6. I.C. injection.
1 . 7. Typical symptoms.
2 . 7. Died.

Mouse No. 14:
24 . 6. I.C. injection.
1 . 7. Typical symptoms. Died.

Mouse No. 15:
24 . 6. I.C. injection.
30 . 6. Typical symptoms. Died.

Mouse No. 33:
2 . 7. I.C. injection.
12 . 7. Nervous symptoms.
13 . 7. Died.

Mouse No. 32:
2 . 7. I.C. injection.
9 . 7. Typical symptoms.
10 . 7. Died.

Mouse No. 33U:
2 . 7. Placed in cage with No. 32.
5 . 7. Symptoms of infection.
6 . 7. Died.
7 . 7. Brain removed and filtered.

Mouse No. 46:
7 . 7. I.C. injection.
18 . 7. Typical symptoms. Died.

Mouse No. 47:
7 . 7. I.C. injection.
18 . 7. Typical symptoms. Died.

I.C. injection = Intracerebral injection.

irritability and exaggerated response to mechanical stimuli. When the skin was pinched or even touched the animal was very restless, jumping across the cage or running rapidly to and fro ; others at this stage were disinclined to move, lying hunched up and immobile in the cage with coat ruffled. A definite arch of the spine in the thoracic region was almost constantly noted, apparently due to a spasm of the flexor muscles. When the animal moved or turned its head this kyphotic attitude persisted.

The *main symptoms* came on rapidly and were fully developed within a few hours. They may be enumerated and described as follows :

Trembling was often seen early as a very fine rhythmical tremor involving the neck, nose and ears. Close observation in a good light was required to detect this tremor, and the rate must have been several times a second. In some cases, later in the disease, a coarse form of trembling appeared which seemed to be associated with loss of equilibration. The animal's body swayed rapidly from side to side.

Unsteadiness was a frequent early symptom, the mouse showing a tendency to fall over on one side. A slight push on one or other side was sufficient to upset such a mouse. This was frequently associated with paresis of one hind limb, so that the animal tended to walk round in circles.

Spasms of muscles.—Besides the hunched back already noted, spasms of muscles in other situations were frequent. Contraction of the neck and thoracic muscles caused the head to be bent forward, the nose being pushed firmly into the meshes of the wire floor of the cage, while the mouse clung with its fore-paws to the wires. Respiratory distress was noted in the later stages, in association with which spasmodic movements of the fore-legs occurred. In some cases spasm of the pectoral and adductor muscles resulted in the fore- and hind-legs being drawn towards the median plane, accentuating further the difficulty in equilibration.

Convulsions of clonic type were seen in some mice. On mechanical stimulation, such as tapping the tail, the animal rolled over and over laterally, suggesting an exaggerated effort to regain the erect posture. *Tonic convulsions* were rare, though some mice, when lifted by the skin of the back, assumed a posture suggesting opisthotonos, the hind-legs being outstretched and the neck arched backwards. An arching forward of the tail until the tip nearly touched the back of the animal's head, was sometimes noted.

While *slight paresis* of one or other limb was frequently an early phenomenon, *complete paralysis* was late and invariably affected the hind limbs first. In such cases the animal used the fore-paws for progression, dragging the hind limbs.

In the concluding stages the mouse lay on one side, making convulsive efforts to regain its feet by feeble rhythmical movements of the legs. Constant dribbling of urine was noted at this stage.

Death was preceded by irregular and convulsive respiration, and the cessation of all limb movements. The mouse lay motionless for some hours before death, though during this time feeble movements could be elicited by any mechanical stimulus applied to the body.

The time from the appearance of the initial symptoms till death in the case of mice inoculated with fresh sheep-brain filtrate was, as a rule, 24–48 hours, though in some cases the condition was protracted up to 4 days.

For comparison, the following extract, describing the *experimental disease in sheep*, is quoted from the paper of Pool, Brownlee and Wilson (1930) already referred to: "In . . . sheep there was a period of incubation of a day or two, followed by a temperature reaction; otherwise the animals apparently remained in perfect health until about the fifth day after inoculation, and then sickened. . . . The earliest symptom, apart from the rise in temperature, was dullness; this was followed by inco-ordination of movement and, frequently, by muscular tremors, chiefly involving the head, by salivation, and by champing of the jaws. Within a few hours or a day or two the animals were unable to stand, but were still able to kick vigorously. At this stage of the disease they lay on one side and were quite unable to change their position. If turned on to the other side they usually struggled until they were replaced in their original position. Coma usually set in soon after this stage had been reached, and death followed within a few hours or a day or two. There was a good deal of variation in the length of the different stages of the illness, and animals which were apparently in normal health at night were frequently comatose or even dead the following morning."

Mice were inoculated with filtrate of sheep brain by the intranasal, intravenous, subcutaneous or intraperitoneal routes, but no distinct evidence of infection was obtained.

PASSAGE EXPERIMENTS.

Passage experiments involving the inoculation of mice with filtrates of the brains of mice dead of the experimental disease have been carried out. The brain of the mouse was ground up with sand and emulsified in 10 c.c. of sterile 0·85 per cent. salt solution. Centrifugalization and the details of filtration are as described for the treatment of sheep brain.

On 4 occasions the condition has been reproduced through three generations of mice without any apparent diminution in virulence, as evidenced by the percentage of successful inoculations, the incubation period, or the severity of symptoms.

CONTROL INJECTIONS.

In order to exclude the possibility of trauma as a factor in the production of symptoms after intracerebral inoculation, 4 mice were inoculated with sterile normal saline. No symptoms resulted over a long period of observation. Further control tests were made in 14 mice, which were inoculated with filtrate of brains of 8 normal mice prepared exactly as in the tests. These animals did not at any time show symptoms referable to the central nervous system.

Mice were inoculated, also, with emulsions of sheep brain obtained from the butcher. In the first experiment of this kind 4 mice were injected with filtrate from one sheep brain and 4 with a similar filtrate from another. After 12 days one animal out of these two groups showed symptoms very similar to those of the louping-ill infection, and it died 2 days later; injections of filtrate of the brain of this mouse were followed by the development of typical louping-ill symptoms in one animal out of two. Distinctive symptoms were not observed in the other 7 mice in the two groups. Since these animals had been kept in the same room with mice infected with material from the brains of louping-ill sheep, and since

contact infection had been shown to occur from animal to animal within the cage (*vide infra*), the experiments with "normal" sheep brain were repeated, isolating the inoculated mice from all other animals, and isolating the separate groups of mice inoculated with different filtrates from one another. In these circumstances, out of 30 mice inoculated with the filtrate of brain emulsion of one or more "normal" sheep, one developed symptoms closely resembling those of louping-ill 6 weeks after injection and it died 2 days after the first signs of illness were seen. Mice inoculated with the brain of this animal have not, to date (after one and a half months), shown any effects of the inoculation. The significance of these findings—2 mice out of 38 showing symptoms of louping-ill after injection with the brain emulsion of sheep exposed for sale as food—is doubtful, but the results are quoted as a contrast to the very high incidence of fatal illness following injection with the brain of sheep known to be infected with louping-ill. The possibility of the virus being present in more sheep than is recognized—possibly in a relation of carrierism or latent in the tissues of the central nervous system—is only mentioned as a question which might be pursued further, since such a condition is found in regard to other neurotropic viruses like that of herpes febrilis.

RESISTANCE TO GLYCEROL.

The resistance of the infective agent to glycerol was not systematically examined. Successful results have been obtained on using sheep brain which had been in 50 per cent. saline-glycerol at 4° C. for periods up to 7 weeks. It has been noted, however, that there is a marked tendency towards lengthening of the incubation period, in some cases the characteristic symptoms appearing only after 2–4 weeks. Associated with this it was observed that in certain cases symptoms appeared in a modified form, sometimes with complete recovery.

"NATURAL" INFECTION.

A fact which points to a high infectivity of the virus was the transmission of the typical disease to uninoculated mice placed in cages with those suffering from the disease. On three occasions this was observed, and in each case the disease was successfully passed to further mice by intracerebral inoculation of brain filtrates. It is evident that in any experimental work on this condition the factor of contact infection must be borne in mind.

CONCLUSION.

These results show how a hitherto somewhat obscure disease of sheep can be transmitted to a laboratory animal, and open the way to more extended study of the disease and its causative virus.

Our thanks are due to Prof. T. J. Mackie for his interest and advice in this work, and to Dr. Russell Greig and Mr. Gordon, of the Moredun Institute of the Animal Diseases Research Association, for the brains of sheep which had died of louping-ill.

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THE GASEOUS REQUIREMENTS OF *BR. ABORTUS* (BOVINE TYPE).

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IN an investigation of certain problems presented by *Br. abortus* it became important to ascertain, in greater detail than had been determined by previous workers (Zwick and Wedemann, 1913; Huddleson, 1921; Smith, 1924; McAlpine and Slanetz, 1928, *b*), the effect of various gases and gaseous mixtures on the growth of this organism.

TECHNIQUE.

Four strains were used, isolated from the blood of patients suffering from undulant fever in this country and in Denmark. All of them were CO_2-sensitive, giving no growth on the surface of dry agar slopes incubated aërobically, but furnishing an abundant growth within two days if incubated in an atmosphere of air, 5 to 10 per cent. of which had been replaced by CO_2.

An agar slope culture, generally 2 to 4 days old, was washed off with 4 c.c. of broth; the suspension was standardized to an opacity of 1000×10^6 *coli*, and diluted 1/350 and 1/123,000 by transferring a 2·9 mm. loopful to two 1 c.c. broth tubes in series. A loopful of the original suspension and of each of the two dilutions was seeded on to agar slopes, free from visible moisture, and of a reaction of pH 7·4–7·6. These slopes were incubated under various gaseous conditions, and the results read at intervals of 2 or 3 days up to a period sometimes reaching three weeks.

The experimental atmospheres were made up in metal jars specially constructed for vacuum work (Wilson, 1928). When anaërobic conditions were required, an electrically heated palladium capsule was employed, similar to that in the usual McIntosh and Fildes jar. Low pressures of gas were measured by a water manometer, higher pressures by a mercury manometer. Exact analyses of the gases in the jar were made, when necessary, in a Haldane apparatus. The cylinder CO_2 used in these experiments contained approximately 98 per cent. carbon dioxide.

RESULTS.

Since about 150 experiments were made with different combinations of oxygen, carbon dioxide, hydrogen and nitrogen, it is frankly impossible to tabulate the results within a reasonable space. The only course, therefore, is to set them out in the form of a summarized verbal description. Certain comments may conveniently be made upon them at this stage.

1. No growth whatever occurs under anaërobic conditions, even in the presence of 5 per cent. CO_2.

2. A lowered oxygen pressure in no way conduces to growth. This finding is in definite opposition to the view that has been widely held since Bang's original paper (1897). Bang's conclusions were based on observations made on shake agar cultures, and, as it is hoped to show in a subsequent paper, his results may be interpreted in a different manner. Nowak's (1908) experiments, in which *Br. abortus* was found to grow satisfactorily in a closed jar containing cultures of *B. subtilis*, have likewise been interpreted as proving that a lowered oxygen pressure favours the growth of *abortus* bacilli. Huddleson (1921), however, has shown qualitatively that CO_2 is evolved from cultures of *B. subtilis*, and in the present series of experiments analysis has demonstrated the presence of as much as 12 per cent. CO_2 in the air over agar slope cultures of this organism. Again, the growth of *Br. abortus* in sealed tubes has led to the conclusion that a lowered oxygen pressure is necessary for growth; but, as the author has shown recently (Wilson, 1930), CO_2 is present in these tubes as the result of flaming the cotton-wool plug, and the insertion of paraffined or rubber corks. So far from a lowered oxygen pressure favouring growth, it actually tends to retard the multiplication of the bacilli (see Conclusion 6).

3. No growth occurs under raised pressures of oxygen, such as 40, 60, 80, or 100 per cent., in the absence of CO_2. This statement, again, is contradictory to the findings of Bang (1897), Preisz (1903), and Holth (1911), all of whom concluded that an atmosphere of more or less pure oxygen was favourable to growth. It may be noted, however, that the first two workers sealed their tubes with melted paraffin, sealing-wax or rubber corks, all of which have been shown to evolve CO_2 (Wilson, 1930), while Preisz grew his cultures in an apparatus having two openings connected by a considerable length of rubber tubing, which in all probability gave off CO_2. It is contended that much the same or even better results would have followed the use of air instead of oxygen; this, indeed, seems probable from the work of Holth.

4. Growth occurs in any pressure of oxygen between about 0·5 per cent. and 99 per cent., provided a minimum of 0·5 per cent. CO_2 is added. The lowest partial pressure of oxygen sufficient to enable growth to occur was not accurately determined, but it is probably under 0·5 per cent.

5. Growth occurs in any pressure of CO_2 between 0·5 per cent. and 98 per cent., provided a minimum of about 0·5 per cent. oxygen is added.

6. Given any partial pressure of CO_2 compatible with growth, maximal growth occurs in 20 per cent. oxygen.

7. Given any partial pressure of oxygen compatible with growth, maximal growth occurs in 5–10 per cent. CO_2.

8. In order to obtain maximal growth, and to ensure that all viable organisms shall develop, the atmosphere in which the cultures are incubated should contain 20 per cent. oxygen and 5–10 per cent. CO_2. Under these conditions the tube seeded from the suspension standardized to an opacity of 1000 million *coli* shows a poorish growth after 24 hours, and a raised confluent growth in 2 days. The tube seeded from the 1/350 dilution shows an effuse semiconfluent growth in 2 days, and a raised semi-confluent growth in 3 days. The tube seeded from the 1/123,000 dilution shows either no growth at all after 2 days, or only very tiny discrete colonies; after about 5 to 7 days, there are usually visible between 100 and 200 colonies, varying from 0·5 to 1·5 mm. in diameter. Departure from these optimum conditions is manifested by a retardation, a diminished luxuriance, or an actual failure of growth, the difference being most noticeable the nearer the extremes of pressure of either gas are approached. Thus in 20 per cent. O_2 + 0·5 per cent. CO_2, or in 95 per cent. O_2 + 5 per cent. CO_2, growth occurs, as a rule, only in the first tube after a delay of 4 or 5 days. When both gases are near the extremes, such as in a mixture of 0·5 per cent. O_2 and 0·5 per cent. CO_2, or 2 per cent. O_2 and 98 per cent. CO_2, growth does not always occur, and when it does, it is very slight and is confined to the first tube.

It is impossible without tabulation of actual results to give any adequate idea of the amount of growth in different combinations of gases, but speaking generally it may be said that in pressures of between 1 and 40 per cent. oxygen and 1 and 30 per cent. CO_2 growth occurs fairly freely, the difference from that in the optimum mixture of 20 per cent. oxygen and 5–10 per cent. CO_2 consisting chiefly in a delayed growth in all tubes, and a diminished number of colonies in the third tube, the colonies which do develop being of definitely smaller size.

9. Neither the addition to the medium of blood, glucose or nitrate will enable growth to occur aërobically in the absence of CO_2, indicating that the factors responsible for the non-development of *Br. abortus* are of a different order from those operative in the metabolism of organisms such as those of the *Pasteurella* group (Webster, 1924, 1925; Schütze and Hassanein, 1929).

It may be added that in the early experiments broth cultures were also put up, but these were discontinued later owing to the fact that the conditions prevailing in a fluid medium are more complex than those on the surface of a solid medium. So far as they went, the results were in accord with those already described; there was a suggestion, however, that a rather lower partial pressure of CO_2 was required for the initiation of growth in broth, but the difficulties of estimating the pressure of CO_2 in the medium itself prevented this being definitely confirmed.

From the foregoing results it is abundantly clear that for the growth of *Br. abortus* both oxygen and carbon dioxide are essential.

THE MODE OF ACTION OF CO_2.

It is clearly of interest to inquire in what way carbon dioxide exercises its peculiarly stimulating effect on the growth of *Br. abortus*. The first explanation that naturally suggests itself, namely that it acts by rendering the medium

acid, is certainly false, since the addition of fixed acid to the medium will not permit growth to occur in the absence of added CO_2.

A more probable explanation is furnished by the work of Jacobs. In aqueous solutions carbon dioxide behaves as a weak acid, dissociating in the following manner:

$$CO_2 + H_2O \rightleftarrows H_2CO_3 \rightleftarrows H^{\cdot} + HCO_3'$$
$$HCO_3' \rightleftarrows H^{\cdot} + CO_3''$$

Working with tadpoles and with various protozoa, Jacobs (1920, *a*) found that a saturated solution of CO_2 was incomparably more toxic than certain mineral and organic acids of the same hydrogen-ion concentration. Evidence obtained from a number of ingenious experiments suggested that the living cell was readily permeable to the undissociated molecule H_2CO_3 and to its anhydride CO_2, while being relatively impermeable to the ions $H^{\cdot}$, HCO_3', and CO_3''. Once the H_2CO_3 molecule had gained access to the interior of the cell, it dissociated so as to give rise to an increase in the intracellular hydrogen-ion concentration, which was responsible for the toxic effects observed. Confirmation of this hypothesis was secured (Jacobs 1920, *b*) by observing that the flowers of *Symphytum peregrinum*, which contain a natural indicator sensitive to alterations in H-ion concentration, changed colour more quickly when placed in a solution of CO_2 in distilled water than in solutions of other acids of equal H-ion concentration. That the effect of the CO_2 was specific was demonstrated by the fact that a similar change in colour could be brought about almost as effectively by exposure to a slightly alkaline solution of CO_2 in M/2 $NaHCO_3$ as to a solution of CO_2 in distilled water, even though the H-ion concentration of the latter solution was approximately four thousand times that of the former.

Shortly after the publication of Jacob's paper, Dale and Evans (1922) demonstrated that CO_2 had a specific effect on the vasomotor system of the cat, independent of the change in the H-ion concentration of the blood which it brought about.

It appears that H_2CO_3 and CO_2 have a specific power of penetrating the living cell-wall, and that it is this power which enables them to give rise to a rapid increase in intracellular acidity. Though in the particular experiments carried out by Jacobs carbon dioxide exerted a toxic effect, it is not unreasonable to suppose that in other instances its rapid diffusion into the cell may have a stimulating action. To test the truth of his hypothesis, experiments were carried out with agar slopes containing sodium bicarbonate. No growth occurred on tubes of this medium incubated aërobically, though it occurred readily in tubes of the same medium incubated in an atmosphere containing 5 per cent. CO_2. It was concluded, therefore, that the bicarbonate radicle could not serve as a substitute for carbon dioxide.

Further evidence in favour of the truth of Jacob's hypothesis is derived from a comparison of the CO_2 requirements of different members of the *Brucella* group. On first isolation and for some generations subsequently, the bovine type of *abortus* will not grow without the addition of CO_2 to the atmosphere (an exception must be made for Rhodesian strains, Bevan, 1930); on the other hand, *melitensis* and the porcine type of *abortus* grow freely under ordinary

aërobic conditions. McAlpine and Slanetz (1928*a*) have shown that, besides this difference in CO_2-sensitivity, the members of the *Brucella* group are likewise distinguished by a difference in their power of utilizing glucose as a source of energy. The bovine type of *abortus* has practically no power of using glucose, whereas *melitensis* and the porcine type of *abortus* can break down 4–18 per cent. of this substance. As Burnet (1930) has pointed out, these observations suggest that the latter two organisms are able by the production of acid from glucose to bring about a rise in intracellular acidity which, in the case of the bovine type of *abortus*, can be brought about only by the presence of a diffusible substance like CO_2 in the gaseous environment. It must, however, be mentioned that the addition of glucose is not essential for the growth of any of these organisms; possibly there is sufficient sugar present in the ordinary heart-extract media to enable the requisite degree of intracellular acidity to be reached.

SUMMARY AND CONCLUSIONS.

1. The bovine type of *Br. abortus* requires both oxygen and carbon dioxide for its development.

2. The optimal concentrations of these two gases are 20 per cent. oxygen and 5–10 per cent. CO_2, the remainder of the atmosphere being constituted by some inert gas such as nitrogen or hydrogen.

3. Contrary to what is frequently stated, a lowered oxygen pressure is less favourable to growth than the pressure of oxygen in air.

4. The *rôle* of CO_2 in promoting growth is discussed, and it is concluded that this substance acts, not by altering the acidity of the medium, but by virtue of its power of diffusing rapidly through the intact cell-wall, and giving rise to an increase in the intracellular hydrogen-ion concentration.

It is a pleasure to express my thanks to Dr. Martin Kristensen for sending me two Danish strains of *Br. abortus*, to Dr. H. H. Dale for referring me to Jacob's original papers, and to the Government Grant Committee of the Royal Society for financial aid in part of this investigation.

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A NOTE ON THE PROPAGATION OF FUJINAMI'S FOWL MYXO-SARCOMA IN DUCKS.

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ATTEMPTS to propagate mammalian tumours in alien species have been made by numerous investigators and have usually been unsuccessful. So far as continued easy propagation is concerned, the attempts have uniformly failed. Partial successes have been recorded by several observers who have inoculated newborn animals with tumour-tissue derived from another nearly related species, but even in these instances success has depended upon the choice of a tumour endowed with unusually great proliferative powers or with unknown qualities which enable cells to multiply in another animal species. The inoculation of newborn animals with any tumour of a nearly related species is therefore not always attended with the partial success which sometimes rewards such experiments, and which tends to support the search for a tumour which will grow equally well in two species. An example of this sort of work is that recorded by myself (1915), in which the polymorph strain of the mouse sarcoma 37 (Imperial Cancer Research Fund) was found to grow relatively easily in newborn rats. Continued transplantation, generation after generation, was not achieved. The success attained depended partly upon the innate qualities of the polymorph sarcoma and partly upon the use of newborn animals. Since the transplanted cells remained mouse-cells, it was scarcely to be expected that continued propagation, such as occurs in the mouse, would be possible.

One of the most remarkable instances of heterologous transplantation is that first recorded by J. B. Murphy (1913), in which foreign cells—mouse, rat, human—are shown to grow and multiply in embryonic chicks. In this instance, also, the transplanted cells remain mouse, rat or human, according to origin; before the chick is born defensive forces come into play by which the alien cells are injured. A few days after birth the tumour usually disappears. Transplantation into mice of a mouse tumour grown in a developing chick is followed by the formation of daughter-tumours, from which further transplantations in mice in an infinite series can be made.

In tumours of fowls in which the causative agent can be separated from the tumour-cells, it has been found that injection of cells or of cell-free filtrates in alien species is not followed by tumour-formation. Peyton Rous inoculated several species of mammals and also pigeons and geese with cells and with filtrates of his tumour No. 1, in each instance with negative results. The

causative agent is thus specific to fowls. I have repeated his experiments on numerous occasions, using mice and rats and ducks, and have always failed to induce tumours. Fujinami has devoted much time to these attempts to propagate fowl tumours in other species of birds, and has reported that after repeated failures he has succeeded in propagating a fowl myxo-sarcoma in ducks for forty generations (1929). It is not clear from the published papers of Fujinami whether the tumour used in these experiments is or is not the original myxo-sarcoma described in 1911. The tumour grows in ducks with remarkable energy, an ordinary graft attaining the size of a "hen's or goose's egg in ten days after transplantation." The tumour, after growing for generations in ducks, can be transplanted again in chickens with uniform success. Fujinami and Hatano conclude that "chicken sarcoma can be converted into perfect duck sarcoma by means of transplantation, and this tumour is endowed with a character common to both kinds of birds."

I have been enabled by the kindness of Prof. Fujinami, who sent me a sample of dried duck tumour, to test these important findings.

The dried tissue was inoculated in chickens on March 10th, 1930. The tissue was reduced to a powder, and then made into a thick paste with distilled water. Two chickens were injected with the paste in the pectoral muscles of both breasts. Eleven days later tumours were observed in both birds. One chicken was killed on April 2nd, 23 days after injection of the paste of dried tissue, and the tumour was transplanted into two other chickens. Since the first transplantation the tumour has been propagated in series in fowls, in which it has grown with a readiness, and with a malignancy, almost equal to that of the Rous sarcoma No. 1. In clinical and biological features, as well as in its histology, Fujinami's tumour is scarcely distinguishable from the Rous spindle-cell sarcoma.

Attempts to propagate the tumour in ducks have been made on a relatively small scale, but, as will be shown, they are sufficiently positive to confirm Fujinami's observations.

The first experiments were made with ducklings of the variety known as "Aylesbury." It was found that a graft of the Fujinami tumour—made into the muscle of the leg—always grew rapidly, forming in 14 days a tumour sometimes larger than a hen's egg. The duckling became helplessly lame as a consequence of the growth. In the Aylesbury breed of ducks, however, the tumour always regressed.

These early results were sufficiently encouraging to warrant attempts with another variety of duck. "Khaki Campbells" were chosen, and it was found that in very young birds of this breed inoculations of Fujinami tumour-cells gave rise to large tumours in 7 to 10 days. The ducklings die from the effects of tumour growth, though so far no metastases have been observed.

It appears to be quite certain that Fujinami is quite correct in his claim that his fowl tumour will grow readily in some varieties of duck, but it is difficult to comprehend that the continued growth of a fowl tumour in another species is dependent upon growth of fowl-cells. The easy filterability of the tumour affords a ready means of testing whether this successful heteroplantation is due to an adaptation of the causative virus to another species of bird.

The protocols of the following experiments illustrate—

(1) That Fujinami's tumour grows in ducklings ;

(2) that the growth is due to the power of the tumour virus to infect duck cells ;

(3) that the tumour in ducks is transplantable in chickens.

On May 16th, 1930, a Fujinami tumour was removed from a chicken (No. 315), minced, and transferred to a duckling (Aylesbury) and to a chick. In both chick and duckling a tumour was palpable on May 23rd. During the following week the tumour in the duckling grew much more rapidly than that in the chick. The duckling became lame and was killed on June 2nd. The tumour was necrotic and hæmorrhagic ; there were no secondary growths.

June 2nd, 1930 : The tumour removed from the duckling was transplanted to a chicken and another Aylesbury duckling. Tumours formed in both birds, but whereas in the chick the tumour grew progressively, in the duck the tumour, after an initial rapid growth, receded and eventually disappeared.

This experience is typical of a series of experiments in the course of which 20 ducklings were used.

June 23rd, 1930 : A routine Fujinami tumour (in a chicken) was taken and 5 gm. were disintegrated by pounding in a mortar with sand. To the mass 100 c.c. of tap-water saline (pH 7·8) was added and thoroughly mixed. The saline extract was spun at 5000 revolutions for 15 minutes, and the supernatant fluid was taken off with a pipette. This fluid was now filtered under gravity through a filter made up of two layers of paper pulp separated by a layer of sand. The filtrate was pale red in colour, clear and sparkling.

This procedure has been found to yield cell-free extracts which, both with the Rous tumour and Fujinami tumour, are exceedingly potent. The proof that this method of filtering extracts of tumours separates the virus from the cells is as follows :

(1) Cells cannot be found in the filtrates.
(2) Filtrates of mammalian tumours prepared in the same way are inactive.
(3) Filtrates of avian tumours are neutralized by antisera.

For example, a pulp-sand filtrate of a Fujinami tumour growing in a duckling is neutralized by an antiserum prepared by injecting a goat repeatedly with cell-free filtrates of Fujinami fowl sarcoma. It is difficult to believe that such an antiserum would be capable of killing duck tumour-cells.

Two chicks and 2 half-grown Aylesbury ducks were inoculated with the filtrate ; the dose in each case was 1 c.c. In the chicks the tumours which formed grew progressively, eventually killing the birds. A tumour formed in one of the ducks only ; it grew rapidly for a week, and then began to recede, finally disappearing in 23 days.

With Aylesbury ducks it has thus been found that Fujinami's tumour will grow for a time, forming large tumours which, however, recede. The formation of tumours after injection of tumour filtrates suggested the possibility that very young ducklings of some other variety might prove more sensitive to the tumour virus.

7th August, 1930 : Tumour tissue from a routine Fujinami tumour was grafted in 2 chicks and 4 young Khaki Campbell ducks ; the dose in each case was 0·05 c.c. Tumours formed in all the birds, those in the 4 ducks growing with much greater rapidity than those in the chicks. Fourteen days after

inoculation all the ducks were lame, due to the large tumours which had formed; one bird was killed and its tumour was excised, and the following procedure carried out:

22nd August, 1930: Two Khaki Campbell ducks were inoculated in the leg with 0·05 c.c. of tumour tissue, and 4 with a paper pulp filtrate of tumour. Tumours formed as a result of all these injections, but in each case after growing rapidly for 10 days began to recede, eventually disappearing.

Further experiments of the same kind convinced me that the Khaki Campbell variety of duck is more sensitive than the Aylesbury, and the following experiments with 4-weeks-old ducklings were therefore carried out.

19th December, 1930: An extract of a Fujinami tumour was made in the manner already described. The extract was spun and the supernatant fluid then filtered through paper-pulp and sand. The filtrate thus obtained was again passed through a second paper-pulp and sand filter. No pressure was used in these filtrations.

The filtrate was injected into three chickens and three ducklings; the dose in each case was 1 c.c.

The chickens developed tumours in the usual way.

The ducklings also developed tumours, which grew with remarkable rapidity. On December 27th, 8 days after injection, 2 of the ducklings were dead and the third was very ill. Examination of the dead ducklings revealed large hæmorrhagic tumours, but no secondary growths. The third duckling was killed and its tumour transplanted to 2 ducklings and 2 chickens. At the same time a filtrate of the tumour was made in the following way: 3 gm. of tissue were broken up with sand and extracted with 100 c.c. of saline. The extract was spun to throw down all tissue *débris*; the supernatant fluid was passed through a Berkefeld candle under a pressure of 20–30 cm. of mercury. Two ducklings were injected each with 1 c.c. of this filtrate. The history of the birds of this experiment is as follows:

(1) The 2 chickens inoculated with tumour tissue from duckling developed tumours which grew and killed the birds.

(2) The 2 ducklings inoculated with tumour tissue from duckling developed tumours which grew very rapidly; one duckling died on January 5th, 1931, 9 days after inoculation, and the other was killed and a filtrate made of its tumour. This filtrate was injected into other ducklings.

(3) The ducklings injected with Berkefeld candle filtrate both developed tumours; one died on the 11th day after inoculation. The tumour in the second bird, after early rapid growth, began to recede and finally disappeared.

The filtrate from the duck tumour mentioned above (2) was injected into two other ducklings, both of which died as the result of the tumours which developed.

The experiments here reported show that the Fujinami fowl tumour is capable of giving rise to tumours in ducks, and that these tumours are transferable to chickens again. In very young ducklings the tumour usually proves fatal. Although the tumour has not been propagated in a long series, the results obtained leave little doubt that Fujinami's claims are correct. In order to establish this beyond doubt, an attempt is now being made to propagate the tumour in series of ducklings.

The facts which distinguish this instance of hetero-transplantation from the partial successes which have been obtained with mammalian tumours are the following: Firstly, in young ducklings the growth of the tumour kills the host; and secondly, there is no loss of growth energy when these duckling tumours are again transplanted in ducklings.

The transmission of the tumour to ducklings by injection of cell-free filtrates demonstrates that the success which attends this hetero-transplantation is dependent upon an adaptation of the tumour's cause to another species of bird, and not upon some peculiarity of the cancer-cell which enables the cell to grow indefinitely in a foreign host. This adaptation of a fowl tumour "agent" is a striking piece of evidence in favour of the view that the "agent" is a virus.

Two curious facts concerning the growth of this tumour in chickens and ducklings deserve attention. First, the tumour grows much faster in ducklings than in chickens; but whereas in chickens retrogression has not been observed, in ducklings it is frequent. The second fact to be noted is that ducklings often die in 8 to 10 days after inoculation. In these cases, although the tumour is large, there is no ulceration, and metastases cannot be found. The duckling becomes ill, and usually within 24 hours of the onset of illness death occurs. Contrasted with this is the fact that chickens usually carry very large tumours, which cause illness and wasting for many days before death supervenes. Whether this rapid death is due to a "toxæmia," or to secondary effects of the immobility of the animal brought about by lameness, cannot yet be decided.

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CULTIVATION OF VACCINIA VIRUS IN A CELL-FREE MEDIUM.

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IN an earlier paper (Eagles and McClean, 1930) it was shown that vaccinia virus could be cultivated in a medium apparently free from tissue-cells. There was, however, a possibility that the medium might contain a small amount of tissue *débris*, and that this favoured increase in virus. Until quite recently it has been generally believed that actively growing tissue is an essential part of any medium for cultivation of this virus. Rivers, Haagen and Muckenfuss

(1929) showed that while the medium used by Maitland and Maitland (1928) did not contain actively growing tissue, the cells remained alive for at least five days, and that this might account for the excellent results obtained by them. It seemed important to investigate the possibility of culturing vaccinia virus in a medium undoubtedly free from tissue-cells. The present communication deals with this problem, and experiments are reported in which filtered tissue extract is employed in the medium.

EXPERIMENTAL METHODS.

Two types of experiments were made, depending upon the presence or absence of rabbit serum in the culture medium. In the first type fresh rabbit serum was used. After the animal had been bled from the heart the kidney was removed aseptically, minced very fine, 3–4 c.c. Tyrode's solution added and the whole centrifuged at high speed for 20 minutes. The supernatant fluid, which we have called "kidney extract," was removed and used as a culture medium. This extract was examined in hanging-drop preparations and in smears for the presence of tissue and was found to be cell-free. No recognizable cell *débris* could be demonstrated, other than an occasional red blood-corpuscle and fat-globule. Kidney extract, virus, serum and Tyrode's solution in amounts giving a final concentration of 1–100 of the virus were mixed and poured in 2 c.c. amounts into "Carrel" flasks. Rivers and Li (1930) had shown that serum was not necessary for the cultivation of vaccinia virus. Therefore in the second type of experiment serum was omitted and sufficient Tyrode's solution added to give the same concentration of virus. A fresh passage strain of neuro-testicular virus was used as seed for the first experiment in each series. All flasks were tested for bacterial sterility by 48-hour cultures in Hartley's broth.

As shown in Table I, excellent increase in virus was obtained in this medium either with or without serum. It was decided, therefore, to control the medium for the presence of cell *débris* as rigidly as possible. Accordingly, after the sixth subculture with serum the medium was filtered through a Chamberland L2 filter. The resulting filtrate was slightly blood-stained and crystal clear. After the fifth subculture without serum the extract was filtered through a Berkefeld "N" filter. No increase in virus was obtained with the "N" filtrate, and therefore a Chamberland L2 filter was used for all subsequent experiments. The extract was diluted with the full volume of Tyrode before filtration, the virus being added just before the inoculum was distributed in the culture flasks.

Generally, flasks were titrated after 2 and 4 days' incubation. In order to discover what length of incubation was most favourable to increase of virus several flasks with 4 c.c. amounts were incubated for 2 to 6 days, and small quantities of fluid were removed for titration each day. The control flask was titrated without incubation.

A heavy deposit usually occurred in the flasks after 24 hours' incubation. This could easily be dispersed by shaking.

Titrations were made intradermally on the shaved back of rabbits, 0·2 c.c. being used for each injection. Readings were made daily from 2 to 7 days.

RESULTS.

The table shows that vaccinia virus can be satisfactorily cultivated in a cell-free medium.

A number of flasks were set up at each experiment, but this table shows only the maximum increase obtained in each set. The flasks of any one experiment, in this as in other methods, showed considerable irregularity of virus increase.

The virus has been carried through seven serial subcultures in the medium containing fresh rabbit serum. When rabbit serum was omitted, the virus was carried through 13 subcultures and an increase of 10^{43} was obtained. The series with the serum was discontinued after the seventh subculture, since its addition was not necessary to virus increase. The table shows that while, generally, a greater increase of virus was obtained when serum was used, there were individual subcultures without serum (*cf.* No. IX) showing a maximum increase. In the one experiment in which a Berkefeld "N" filter was used, no increase in virus occurred.

Satisfactory cultivation of virus was obtained when the extract, either with or without rabbit serum, was passed through a Chamberland L2 filter.

Cultivation of virus has been carried through four generations in subculture, using a Chamberland L2 filtrate of kidney extract without rabbit serum. Virus from the eighth subculture in unfiltered extract without serum was used as seed for the initial experiment of this series.

From time to time, as shown in the table, neutralization of the culture virus by anti-vaccinal serum was tested. This was always successful.

Different rabbits have shown considerable variation in reaction to the same sample of culture virus. Retitration of an apparently negative sample of virus has given excellent reactions on another rabbit, and it has been considered necessary to control this variation by an intradermal injection of 1 : 100 calf lymph in all rabbits used for titration. In our experience rabbits which react atypically to calf lymph do so to culture virus. In our previous investigations with the method of Carrel and Rivers, and with that of Maitland and Maitland, we reported considerable irregularity of virus increase; we did not then appreciate the extent of variation in rabbit response, and this may partially explain the apparent irregularity obtained by all three methods.

The period of maximum growth of virus varied from 2 to 6 days in different experiments, and it has not been possible to obtain a constant growth curve. Attempts to measure the daily increase were complicated by the rabbit variation referred to above.

We have found it important when subculturing to continue in series regardless of small amounts of increase in virus. As shown in the table, the sixth subculture without serum gave only slight virus increase, while subculture from this gave excellent results in the seventh and eighth generations.

A strain of neuro-testicular virus which had recently been passaged was used as the initial seed for both series of experiments. It was decided to defer investigation of the behaviour of a dermal strain of virus.

Table showing Cultivation of Vaccinia Virus in Kidney Extract, With and Without Serum, and in Filtered Kidney Extract.

CULTURES WITH SERUM.

		In unfiltered extract.	Extract filtered through L2 Chamberland candle.
I.	30.ix.30	Virus : Fresh testicular passage. Control flask = 10^{-6}. Culture = 10^{-6}.	
II.	23.x.30	Control = *nil*. Culture = 10^{-7}.	
III.	30.x.30	Control = *nil*. Culture = 10^{-7} (neutralized by A.V. serum).	
IV.	6.xi.30	Control = 10^{-1}. Culture = 10^{-4}.	
V.	20.xi.30	Control = *nil*. Culture = 10^{-5}.	
VI.	4.xii.30	Control = Doubtful. Culture = 10^{-3} (neutralized by A.V. serum).	
VII.	11.xii.30	Control = 10^{-6}. Culture = 10^{-3} (neutralized by A.V. serum).	
VIII.	17.xii.30	Control = 10^{-1}. Culture = 10^{-6}.	17.xii.30 . Control = *nil*. Culture = 10^{-7}.

Result.—Seven subcultures : Approximate virus increase = 10^{27}.
,, dilution of original virus = 10^{-16}.

CULTURES WITHOUT SERUM.

		In unfiltered extract.	Extract filtered through L2 Chamberland candle.	Extract filtered through Berkefeld N.
I.	28.x.30	Virus : Testicular passage. Control flask = *nil*. Culture = 10^{-6}.		
II.	4.xi.30	Control = 10^{-1}. Culture = 10^{-4}.		
III.	11.xi.30	Control = *nil*. Culture = 10^{-3}.		

III.	11.xi.30	Control = *nil.* Culture = 10^{-2}.				
IV.	25.xi.30	Control = 10^{-3}. Culture = 10^{-5}.				
V.	2.xii.30	Control = 10^{-5}. Culture = 10^{-6}.				
VI.	10.xii.30	Control = 10^{-6}. Culture = 10^{-8} (neutralized by A.V. serum).				
VII.	15.xii.30	Control = *nil.* Culture = 10^{-3}.			15.xii.30	Control = *nil.* Culture = *nil.*
VIII.	30.xii.30	Control = 10^{-5}. Culture = 10^{-6}.				
IX.	8.i.31	Control = *nil.* Culture = 10^{-6}.	8.i.31	Control = *nil.* Culture = 10^{-2}.		
X.	22.i.31	Control = 10^{-4}. Culture = 10^{-6}.	22.i.31	Control = Doubtful. Culture = 10^{-6}.		
XI.	29.i.31	Control = 10^{-5}. Culture = 10^{-7}.	29.i.31	Control = 10^{-2}. Culture = 10^{-6}.		
XI*a*.	5.ii.31	Control = 10^{-6}. Culture = 10^{-7}.			5.ii.31	Control = 10^{-6}. Culture = 10^{-6}.
XII.	12.ii.31	Control = 10^{-6}. Culture = 10^{-7}.			12.ii.31	Control = 10^{-2}. Culture = 10^{-7}.
					19.ii.31	Control = 10^{-8}. Culture = 10^{-6}.
					26.ii.31	Control = *nil.* Culture = 10^{-4}.

Result.—Thirteen subcultures : Approximate virus increase = 10^{43}.
,, dilutions of original virus = 10^{-26}.

Explanation.—Control = titre of inoculum before incubation. Culture = maximum titre of virus obtained after incubation. A.V. serum = antivaccinal serum.

CONCLUSIONS.

Attempts to cultivate vaccinia virus in media devoid of living tissue have until now been unsuccessful, although many types of artificial media have been investigated. Until the work of Maitland and Maitland, active proliferation of tissue-cells was thought to be essential to growth of virus. Their work established the fact that this was not so. Rivers, Haagen and Muckenfuss offered what seems an adequate explanation of the success of the Maitland medium by showing that the kidney-cells remained alive for at least 5 days. It seemed important to discover whether the presence of tissue-cells, whatever their state, was essential to the cultivation of vaccinia virus. In our earlier work with kidney extract where centrifugalization was employed to obtain a cell-free medium, it was possible that occasional cells might persist, since smears and hanging-drop preparations do not necessarily represent the state of the medium as a whole. In order to make quite certain that the medium contained no trace of tissue-cells, filtration was resorted to. Results with this filtered medium show quite clearly that it is possible to cultivate the virus in an entirely cell-free medium and to propagate it in serial subculture.

It is not, at present, clear why this cell-free medium should promote the growth of vaccinia virus while other artificial media do not. It is possible that the explanation lies in the use of kidney extract in a fresh condition, although some preliminary experiments, in which an extract which had been kept for 48 hours in the cold room was used, would indicate that freshness of the extract may not be the essential factor. Further work with extracts kept for longer periods under varying conditions is indicated. The presence of a cloudy deposit after the extract has been incubated for a short time suggests that some substance is present which may not be contained in ordinary artificial media. Adaptation of the virus to the medium does not seem to be important, since excellent increase was obtained in a primary culture when a fresh testicular virus was used as seed.

The variation in reaction of individual rabbits to vaccinia virus is important, since it might be, to some extent at least, an explanation of irregularity in virus increase in the flasks of an experiment. While virus grown in cell-free extract and in other media is apparently unchanged in its characteristics, it seems advisable to control the reaction of rabbits with a non-culture strain of virus. Commercial calf lymph has been used in our work for this purpose. Several instances appear in the table where a culture after dilution for subculture purposes gave a higher titration than before dilution, although it had been stored for some days in the cold room. In view of the fact that a culture may show wide variation in titre when tested on different rabbits, this is not surprising.

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THE CONSERVATION OF VACCINIA VIRUS GROWN *IN VITRO*.

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A STUDY of the cultivation of vaccinia virus in chick embryo tissue (Eagles and McClean, 1929) had shown that virus obtained by this method deteriorated rapidly at cold room temperature (± 1° C.). Subsequently Maitland and Laing (1930) reported that virus cultivated in rabbit kidney and serum could be preserved for considerable periods in the cold room. Our own preliminary observations with virus cultivated by their method had suggested that this virus kept more satisfactorily than that grown in chick embryo tissue. There was, however, considerable variation in the rate of deterioration of different samples stored under apparently identical conditions. This experience confirms that of Maitland and Laing, since reference to their article shows that whereas one virus originally infective in a dilution of 1 in 78,125 was still infective at 1 in 3125 after 358 days' storage, the infectivity of another sample had dropped from 1 in 3125 to 1 : 625 in 7 days ; their table shows other examples of similar variations.

It was of interest and of practical importance to determine the conditions under which culture virus could be preserved, to compare the results of storage by different methods and to control the variable deterioration of different samples of the same virus.

METHODS AND MATERIAL.

A considerable series of tests were made with vaccinia virus from various sources under different conditions of storage. The viruses employed were diluted commercial calf lymph, diffusates from passage in the rabbit dermis, virus of both dermal and testicular origin cultivated in chick embryo and plasma and in the rabbit kidney and serum medium of Maitland and Laing ; latterly testicular virus cultivated in the cell-free kidney extract with and without serum (Eagles and McClean, 1931) was used.

The substances whose protective effect was investigated were cysteine hydrochloride, glycerine (50 per cent. and 90 per cent.), agar (0·25 per cent.), gelatine (0·125 per cent.), borax buffer solution (Glenny, Pope and Waddington, 1928), the addition of fresh minced tissue, Hartley's broth and "hormone" broth (Ward, 1929).

The viruses mixed with these substances were stored at room temperature, in the cold room (±1° C.) and frozen at −18° C. The effect of oxidation in

the presence of the above substances was investigated; some samples were sealed with vaseline or stored in sealed capillaries and ampoules; others were stored anaërobically in modified McIntosh and Fildes' jars, satisfactory anaërobiosis being controlled by reduced methylene-blue. The effect of varying the pH of the virus suspensions and of drying *in vacuo* were also investigated.

Since culture virus is occasionally contaminated by bacteria, some samples of virus were stored with 1 per cent. phenol in order to determine whether this substance exerted any deleterious effect on the conservation of the virus. Stored viruses were re-titrated intradermally in rabbits over periods varying from 42 to 218 days.

RESULTS.

Since most of the above methods did not preserve the virus sufficiently to be of practical importance, it is not proposed to give the results of all these experiments in detail. It will suffice to summarize the information obtained.

Different samples of vaccinia virus, whether obtained by cultivation or passage, show great variation in their rate of deterioration when stored under apparently identical conditions. The addition of glycerine to the virus has shown no material protective effect in many experiments. The addition of "hormone" broth slightly delayed deterioration, but our experiments with culture virus did not suggest that the protection afforded was sufficient to have a practical application. The result of storing virus in strict anaërobiosis at room temperature showed that oxidation was not the only factor involved since deterioration was only slightly delayed; nevertheless, anaërobic storage in the cold room, when compared with the result of aërobic storage at the same temperature, did delay considerably the deterioration of all types of virus. The effect of anaërobiosis will be discussed more fully later. Storage at $-18°$ C., even with the addition of glycerine to prevent freezing, had little conserving effect. It appeared that the addition of 0·25 per cent. agar exerted a definitie protective effect, but was not in itself sufficient to maintain a culture virus at high titre over considerable periods. The other preservatives investigated either failed to protect the virus, or their effect was so feeble as to be of no practical use.

The addition of 1 per cent. phenol under various conditions of storage in the cold room did not reduce the infectivity of the virus over periods as long as 209 days. If the virus was frozen in the presence of phenol, however, it was destroyed. This effect of phenol in the frozen mixture is similar to that which occurs when phenolized diphtheria antitoxin is frozen.

No satisfactory method of preserving virus cultivated in chick embryo and plasma was discovered. It appeared from our experiments that storage in the cold ($\pm$ 1° C.) under anaërobic conditions in a solution of 0·25 per cent. agar was the best method available. Unfortunately it was impossible to make an extensive trial of this method, since the use of this medium had been discontinued.

Anaërobic cold room storage of virus cultivated in chick embryo and plasma and in Maitland and Laing's medium gave interesting results. Table I illustrates the effect of storing chick embryo culture virus anaërobically. Anaërobiosis delays the deterioration of this virus slightly, but the protection afforded is not sufficient or regular enough to be of practical utility.

TABLE I.—*Virus Cultivated in Carrel and Rivers's Chick Embryo Medium Stored Anaërobically.*

Source of virus.	Original titre.	1st retitration, 2.i.30.	2nd retitration, 2.ii.30.	3rd retitration, 2.iv.30.	Total length of storage.
Culture, 30.xi.29, 1	10^{-6}	10^{-3}	10^{-3}	10^{-2}	124 days (phenol 1%).
,, ,, 2	10^{-5}	10^{-1}	..	..	34 ,,
,, 29.x.29, 1	10^{-4}	10^{-1}	..	..	65 ,,
,, ,, 2	10^{-4}	$0{\cdot}2\times10^{-3}$	$0{\cdot}2\times10^{-3}$	10^{-1}	155 ,,
,, 11.x.29	$0{\cdot}2\times10^{-4}$	10^{-3}	..	..	82 ,,
,, 2.xii.29	10^{-6}	10^{-5}	10^{-4}	10^{-1}	121 ,, (phenol 1%).
,, 23.ix.29	10^{-4}	10^{-2}	..	..	101 ,,

Table II shows the result of storing 19 samples of virus obtained from Maitland's medium aërobically in the cold room. It will be seen that although this virus will survive over considerably longer periods than that obtained from chick embryo cultures, the rate of deterioration under these conditions is irregular. In our experience the viability of the virus seemed to be fairly constant for the different flasks of any one experiment, but showed gross variation in samples from different experiments, notwithstanding the use of apparently identical methods and material.

TABLE II.—*Virus Cultivated in Maitland's Medium Stored Aërobically in Cold Room.*

Source of virus.	Original titre.	Retitration.	Length of storage.
Maitland's culture virus V. 151. Received 14.xi.29	10^{-6}	10^{-6}	218 days.
Dermal culture virus 1, 18.ii.30	10^{-6}	10^{-5}	122 ,,
,, ,, 2	10^{-6}	10^{-6}	
Testicular culture virus, 23.iii.30	10^{-5}	10^{-5}	88 ,,
Dermal culture virus 1, 12.iv.30	10^{-6}	10^{-6}	69 ,,
,, ,, 2	10^{-6}	10^{-6}	
,, ,, 3	10^{-4}	10^{-4}	
,, ,, 4	10^{-2}	10^{-2}	
Testicular culture virus 1, 12.iv.30	10^{-6}	10^{-4}	69 ,,
,, ,, 2	10^{-6}	10^{-3}	
,, ,, 3	10^{-6}	*Nil*	
Testicular culture virus 1, 20.v.30	10^{-4}	10^{-1}	31 ,,
,, ,, 2	10^{-6}	*Nil*	
,, ,, 3	10^{-6}	,,	
Dermal culture virus 1, 20.v.30	10^{-5}	10^{-2}	31 ,,
,, ,, 2	10^{-6}	10^{-5}	
,, ,, 3	10^{-6}	*Nil*	
Testicular culture virus 1, 2.vi.30	10^{-3}	,,	18 ,,
,, ,, 2	10^{-6}	,,	

Table III shows the result of storing 11 samples of virus cultivated in Maitland's medium under complete anaërobic conditions in the cold room. Repeated titrations of these viruses were made over periods ranging from 1 to 7 months. The effect of anaërobiosis upon the preservation of Maitland culture virus is in striking contrast to its effect upon virus cultivated in chick embryo medium. Many of the samples shown in Table III maintained their full titre over the whole period, while the deterioration of other samples is

TABLE III.—*Virus Cultivated in Maitland's Medium Stored Anaërobically in Cold Room.*

Source of virus.	Original titre.	1st retitration, 2 . i . 30.	2nd retitration, 5 . ii . 30.	3rd retitration, 3 . iv . 30.	4th retitration, 17 . vi . 30.	Total length of storage.
Culture 20.xi.29, 1	10^{-6}	10^{-6}	10^{-6}	10^{-5}	10^{-6}	209 days (phenol 1%).
,, ,, 2	10^{-6}	10^{-6}	10^{-6}	10^{-5}	10^{-6}	209 ,, ,,
,, 4.xii.29, 1	10^{-9}	10^{-5}	10^{-6}	..	..	63 ,,
,, ,, 2	10^{-8}	10^{-5}	10^{-3}	10^{-5}	10^{-5}	205 ,,
,, 21.xi.29, 1	10^{-6}	10^{-6}	10^{-5}	10^{-6}	..	133 ,,
,, ,, 2	10^{-6}	10^{-6}	10^{-5}	..	..	76 ,,
,, 27.xi.29, 1	10^{-7}	10^{-8}	10^{-7}	10^{-7}	10^{-6}	202 ,, (phenol 1%).
,, ,, 2	10^{-7}	10^{-8}	10^{-8}	*10^{-7}	..	127 ,,
,, 10.xii.29,1	10^{-6}	10^{-6}	10^{-6}	**10^{-2}	..	114 ,,
,, ,, 2	10^{-7}	10^{-6}	10^{-6}	10^{-6}	10^{-6}	189 ,, (phenol 1%).
,, date unknown	..	10^{-6}	10^{-7}	10^{-6}	..	*Circa* 110 days

* Owing to shortage of fluid, 1 c.c. of phosphate solution was ground up with the mush and this titrated.
** Very small quantity of fluid left.

TABLE IV.—*Virus Cultivated in Cell-free Medium Stored Anaërobically in the Cold Room.*

Source of virus.	Original titre.	Retitration.	Length of storage.
Cultures with serum :			
Culture, 2.x.30, 1	10^{-8}	10^{-6}	131 days.
2	10^{-8}	10^{-5}	131 ,,
,, 29.x.30, 1	10^{-7}	10^{-3}	104 ,,
2	10^{-7}	10^{-6}	104 ,,
3	10^{-4}	10^{-6}	110 ,,
4	10^{-5}	10^{-5}	110 ,,
,, 3.xi.30, 1	10^{-7}	10^{-4}	106 ,,
2	10^{-7}	10^{-4}	106 ,,
3	10^{-7}	10^{-4}	109 ,,
,, 10.xi.30	10^{-6}	10^{-4}	102 ,,
Cultures without serum :			
Culture, 1.xi.30, 1	10^{-4}	10^{-5}	138 ,,
2	10^{-6}	10^{-6}	138 ,,
,, 27.xi.30, 1	10^{-5}	10^{-5}	82 ,,
2	10^{-5}	10^{-4}	82 ,,
,, 12.xii.30, 1	10^{-8}	10^{-5}	70 ,,
2	10^{-8}	10^{-5}	70 ,,
Cultures in filtered extract :			
Culture, 19.xii.30	10^{-7}	10^{-1}	62 ,,
,, 26.i.31, 1	10^{-6}	10^{-6}	32 ,,
2	10^{-6}	10^{-5}	32 ,,
,, 3.i.31	10^{-6}	10^{-3}	27 ,,
,, 9.ii.31, 1	10^{-7}	10^{-4}	16 ,,
2	10^{-6}	10^{-2}	16 ,,
,, 14.ii.31	10^{-7}	10^{-2}	13 ,,

comparatively slight. It will be noticed that 4 samples had been sterilized by the addition of 1 per cent. phenol; this showed no deleterious effect upon the virus. It would appear from this experiment that culture virus may be satisfactorily preserved in 1 per cent. phenol under anaërobic conditions at a temperature of ± 1° C.

During our experiments with the cultivation of virus in cell-free kidney extract (see Eagles and McClean, 1931, *b*) all cultures were stored anaërobically in the cold room. Table IV illustrates the results obtained on retitration of a representative group of these viruses after varying periods of storage.

The virus appears to survive for a considerable period without great loss in titre, whether or not the culture medium contains fresh rabbit serum, when the kidney extract has not been filtered. Since experiments using filtered kidney extract have been carried out more recently than with unfiltered extract, the length of storage is not strictly comparable and the series is more limited. In filtered extract, however, the virus appears to deteriorate more rapidly than in unfiltered when conserved under similar conditions. Comparison of Table III and Table IV shows that under similar conditions of storage vaccinia culture virus survives better when grown and stored in Maitland's kidney medium than in cell-free kidney extract. This is possibly accounted for by the high protein content of the Maitland medium.

CONCLUSIONS.

1. Glycerine has little effect in conserving vaccinia virus, whether this is obtained by culture or from animal passage.

2. No satisfactory method of preserving virus cultivated in the chick embryo and plasma medium of Carrel and Rivers has been discovered. Anaërobic storage in the presence of 0·25 per cent. agar at ± 1° C. gave better results than other methods.

3. One per cent. phenol may be used to free culture virus from bacterial contaminants without any deleterious effect upon the virus over prolonged periods of storage provided the medium is not frozen.

4. Virus cultivated in the kidney medium of Maitland and Laing may be satisfactorily preserved in the cold room under anaërobic conditions.

5. Virus cultivated in unfiltered cell-free kidney extract survives without great loss in titre under anaërobic conditions in the cold room.

6. Filtered cell-free kidney extract is a less satisfactory medium than unfiltered for preserving vaccinia culture virus under similar conditions.

7. Virus cultivated in Maitland's kidney medium survives better than in cell-free kidney extract under anaërobic conditions in the cold room.

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RESISTANCE TO THE GROWTH OF TRANSPLANTABLE TUMOURS: (2) THE INFLUENCE OF DYESTUFFS AND OF COLLOIDS ON NATURAL RESISTANCE.

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In a former paper (Ludford, 1931) evidence has been brought forward that the immunity induced in mice by embryo skin to the subsequent transplantation of tumours can be broken down by vital staining with trypan blue. In that paper I referred to the fact that transplantable tumours of mice often appear to grow better when the animals are injected with dyestuffs or metallic colloids. The present communication is devoted to the description of experiments carried out to investigate this aspect of tumour growth under controlled conditions.

DYESTUFFS AND COLLOIDS AS CHEMOTHERAPEUTIC AGENTS FOR CANCER.

Beneficial results in the treatment of human cancer cases have been claimed at different times for colloids of copper, iodine, sulphur, selenium, lead, and the acid dyestuff isamine blue. Animal experiments have failed to indicate that any of these has a direct action upon the cancer-cell. In an earlier paper (Ludford, 1929) I suggested that any possible action might be due to a localized accumulation of the colloid, or dye, brought about by macrophages which happened to be present in or around the tumour.

Large numbers of dyestuffs have been injected into tumour-bearing mice without any suggestion of their inhibiting growth. Thus Weil (1916) injected into tumour-bearing rats over 20 dyes of the benzidene group, and Marsh and Simpson (1928) investigated the action on mouse tumours of more than a hundred aniline dyes. Munck (1928) thought trypan blue retarded the growth of transplantable carcinoma and sarcoma of the mouse, but I did not find it had any appreciable influence on the growth of a well-established tumour (Ludford, 1929). Lignac and Kreuzwendedich von dem Borne (1928) describe trypan blue as having just the opposite action upon tumour growth. They say "die chronische Vitalfärbung der Mäuse mit Trypanblau macht sie zu einem günstigen Entwicklungsboden für die Sarkomzellen." The results of experiments described in this paper suggest that relatively large injections of dyestuffs and colloids favour tumour growth by lowering the natural resistance of the organism.

FACTORS WHICH INFLUENCE THE GROWTH OF TRANSPLANTABLE TUMOURS.

Experiments concerned with the growth of transplantable tumours are influenced by two factors, or sets of factors. These are: (1) The biological state of the cells of the graft. Although what is apparently healthy tumour is inoculated into mice at each transplantation, the results vary from time to time. The tumour strains pass through periods of depressed growth. It has been my experience that these most frequently follow a phase of intensive growth.

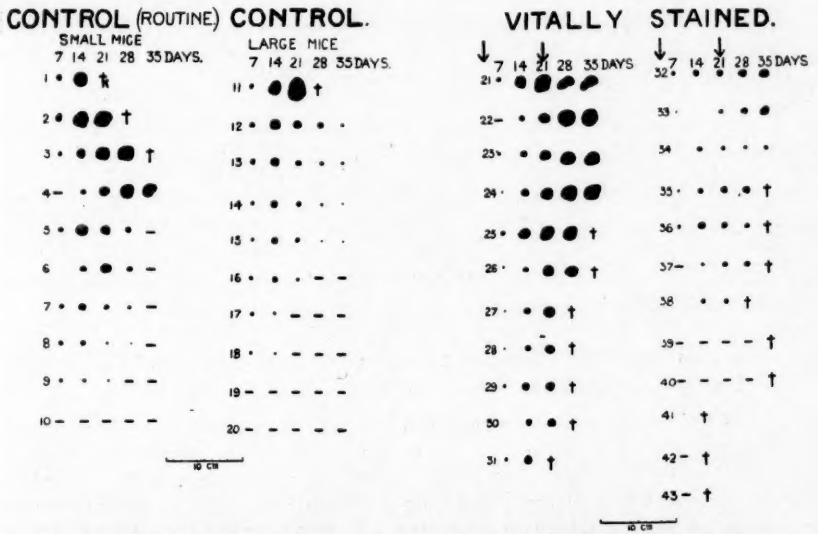

FIG. 1.—Growth of the tumour 206/532A as indicated by weekly chartings from the time of transplantation in small and large controls, and in large mice vitally stained with trypan blue. (Arrows indicate the times of the first and last injections of the series. K denotes killed. † died.)

(2) The resistance which the organism offers to the growth of the graft. The indications are that different strains of mice vary in their degree of resistance, and individual variations also occur. The various transplantable tumours also vary in the extent to which they induce resistance.

The general results of my experiments have been that when tumours are growing at their maximum rate, injections of dyestuffs and colloids in non-lethal doses are without influence on tumour growth. In those cases, however, in which the animal is resistant, as indicated by a preliminary growth of the graft followed by regression, dyestuffs and colloids favour growth by their action upon the mechanism of resistance. Three experiments have been selected for description from many to illustrate this breaking down of natural resistance.

INFLUENCE OF TRYPAN BLUE (GRÜBLER) UPON RESISTANCE TO THE GROWTH OF TUMOUR 206 OF THIS LABORATORY.

The transplantable carcinoma 206 exhibits a marked tendency to undergo spontaneous regression. This occurs most frequently when relatively large pieces of tumours are inoculated into large mice. My experiments have been carried out by injecting such mice with dyestuffs, and observing the influence on the rate of growth of the tumours. The following experiment illustrates the action of vital staining with trypan blue on reabsorption.

Experiment 1. (Fig. 1.)

1st day of experiment.—Ten small mice (average weight 17 gm.) and 33 large mice (average weight 25 gm.) were inoculated by needle, in the right axilla, with approximately 0·02 gm. of tumour 206/532A.

The 10 small mice (Nos. 1–10) and 10 of the larger ones (Nos. 11–20) were set aside as controls. The remainder of the large mice (Nos. 21–43) received subcutaneous injections, on the back, of an 0·5 per cent. solution of trypan blue (Grübler) in distilled water as follows :

2nd day of experiment.—1·0 c.c.

5th, 6th, 7th, 13th and *18th days of experiment.*—0·5 c.c.

The growth of the tumours is represented in Fig. 1. The arrows at the top indicate the period during which the injections were given. More injections were not possible owing to the toxic action of the dyestuff.

The figure shows that there was considerable reabsorption even in the small mice. All but one of the tumours in the large control mice (No. 11) regressed ; but the tumours in the large vitally stained mice grew much better.

INFLUENCE OF VITAL NEW RED (GRÜBLER) UPON RESISTANCE TO THE GROWTH OF TUMOUR 206.

Vital new red is less toxic than trypan blue, but less suitable as a vital dye owing to the difficulty in retaining the granular staining in permanent preparations.

Experiment 2. (Fig. 2.)

1st day of experiment.—Ten small mice (average weight 16 gm.) and 29 large mice (average weight 25 gm.) were inoculated by needle in the right axilla with approximately 0·02 gm. of tumour 206/542B. As in the previous experiment, the 10 small mice (Nos. 1–10) and 10 of the larger ones (Nos. 11–20) were set aside as controls ; the remainder (Nos. 21–39) received subcutaneous injections on the back of 0·5 per cent. solution of vital new red (Grübler) in distilled water on the following days, 2nd, 4th, 6th, 8th, 12th, 16th, 18th and 20th.

It will be seen from Fig. 2 that the tumour was growing badly at the time of the experiment. In the large control mice there was not one progressively growing tumour ; but in the large vitally stained mice growth was better than in the small routine ones. The peculiar growth in this experiment suggests that while the tumour was passing through a period of depression the mice also exhibited considerable resistance to its growth.

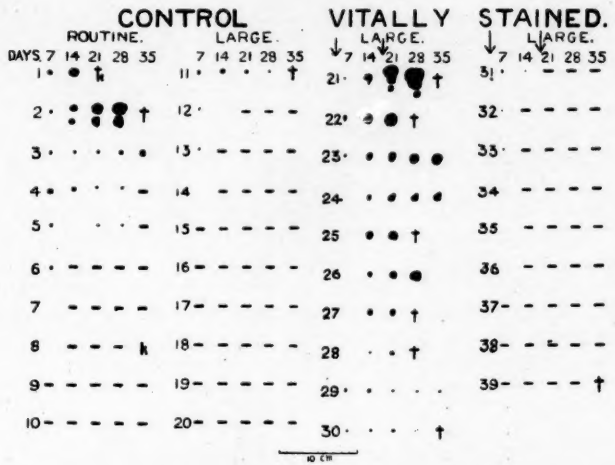

FIG. 2.—Growth of the tumour 206/542 B as indicated by weekly chartings in small and large controls, and in large mice vitally stained with vital new red.

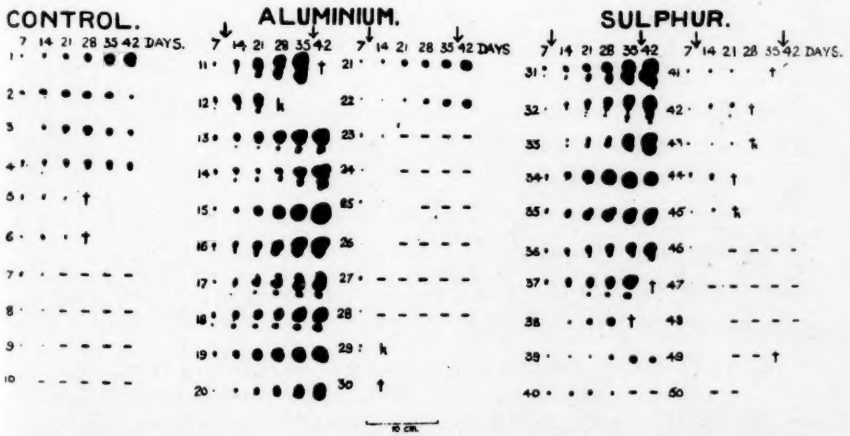

FIG. 3.—Growth of the tumour Twort 204 C in controls and in mice which have received intravenous injections of colloids of aluminium hydroxide and sulphur.

INFLUENCE OF METALLIC COLLOIDS UPON RESISTANCE TO THE GROWTH OF TWORT'S CARCINOMA.

A large number of experiments have been carried out with colloids of lead, sulphur, aluminium and bismuth. The following experiment illustrates the influence that such colloids may exercise in breaking down resistance to tumour growth:

Experiment 3.

Fifty mice were inoculated by needle in the right axilla with approximately 0·02 gm. of Twort's carcinoma 204 C. Ten mice were set aside as controls. The others were divided into two batches of 20, one subsequently receiving injections of colloidal aluminium hydroxide, the other of colloidal sulphur. For the colloids used in these experiments I am indebted to Dr. A. T. Todd, of Bristol University. For the first injections the colloids were diluted with normal saline. The concentration of the injections was gradually increased until the limit of toxicity was reached. Each time 0·4 c.c. of fluid was injected into the tail vein. The injections were carried out as follows:

9th	day	.	.	3	Al	:	10	saline	.	.	3	S	:	10 saline
13th	,,	.	.	2½	Al	:	6	,,	.	.	2½	S	:	6 ,,
18th	,,	.	.	2½	Al	:	6	,,	.	.	2½	S	:	6 ,,
22nd	,,	.	.	4	Al	:	4	,,	.	.	4	S	:	4 ,,
26th	,,	.	.	6	Al	:	4	,,	.	.	6	S	:	2 ,,
32nd	,,	.	.		Al				.	.				S
37th	,,	.	.		Al				.	.				S

Al denotes aluminium hydroxide colloid, and S sulphur colloid. The figures before the letters denote the number of parts of each in the fluid injected.

Fig. 3 shows that while there was a good deal of reabsorption in the controls, the tumours of the injected mice grew much better.

PROPERTIES OF THE SUBSTANCES USED FOR LOWERING RESISTANCE.

These experiments show that substances of very different physical and chemical properties are capable of breaking down the resistance of mice to the growth of transplantable tumours. The substances used, however, have two properties in common. They are segregated by certain cells of the animal body, especially the macrophages, also in the doses employed they reached the limit of toxicity. Experiments are still in progress to determine how far either, or both, of these properties of the dyestuffs and colloids are responsible for the results obtained.

CONCLUSION.

By vital staining with acid dyes (trypan blue, vital new red) and by the intravenous injection of inorganic colloids it is possible to lower the resistance of mice to the growth of transplantable tumours.

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SOME OBSERVATIONS ON CALCIUM AND PHOSPHORUS IN THE BRAIN IN DIFFERENT CONDITIONS.

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IT is common for conclusions to be drawn from histological studies of the brain in diseases of the central nervous system as to the presence of abnormal deposits of calcium, but these conclusions have rarely been controlled by chemical analysis. That calcium has physiological significance in the normal functioning of the nervous system is generally recognized. Macallum and Voegtlin (1909) found diminished amounts of calcium in the brain as well as in the blood in tetany. Critchley and O'Flynn (1924) determined the amount of calcium in the blood and cerebro-spinal fluid in a number of cases of nervous disease and found low figures in tetany, but never in either fluid increased amounts. And yet increased amounts of calcium may be expected to be significant, since Sabbatani (1901) found that the application of calcium salts depressed the excitability of the cerebral cortex, and on the contrary the removal of them, assumed to occur when citrate or oxalate solutions were applied, increased it. Demole (1927) injected calcium salts into the hypothalamic region in animals and induced disturbance of sleep.

But the histological methods for the detection of calcium either in the walls of vessels or elsewhere in the central nervous system, on which in the past reliance has been placed, are not reliable. Calcium sulphate crystals may be unmistakable, but when identified point to such abnormal amounts of calcium

as can have no functional significance other than that of foreign bodies. The purpurin test for calcium is, in my experience, no more satisfactory than older ones. I have found by chemical analysis as much as six or seven times the usual amount of calcium in the cortex of the brain without being able to get any histological evidence of its presence whatever. The fact, indeed, that iron, for which the micro-chemical tests are delicate and reliable, can be demonstrated in deposits that would have formerly been described simply as calcareous, has led to a tendency to regard such deposits as composed of iron salts rather than calcium; and yet in some instances the presence of calcium has been definitely identified (Eaves, 1926; Da Fano and Perdrau, 1927), and in such cases iron may not be found.

The conditions in which, according to general principles of pathology, calcification is prone to occur are, apart from the hyaline degeneration seen in arterio-sclerotic vessels, those in which a degenerative change in cells leads to the liberation of higher fatty acids and the precipitation of their calcium soaps and conditions in which calcium salts are through some disturbance of calcium metabolism present in the blood and body fluids in larger amounts than normal, and there is consequently a tendency for the precipitation of calcium phosphate and carbonate. Now, although there is no nervous disease, as already mentioned, that has been found to be associated with a high blood-calcium, the elements of nervous tissue are, on the other hand, rich in complex compounds of higher fatty acids, and degenerative change in this tissue is a probable source of calcium soaps. Moreover, since such degenerative change may be accompanied by loss of phosphorus (Mott and Barratt, 1899, degenerated tracts of cord; Mott and Halliburton, 1901, general paralysis; Lorrain Smith and Mair, 1911, general paralysis and hemiplegic softening; and Pighini, 1912, 1914, 1921, general paralysis and dementia præcox), phosphoric acid liberated in the degeneration may be expected to lead to precipitation of calcium phosphate, side by side with calcium soaps.

The unreliability of histological methods for detecting variations in the amount of calcium in the brain makes it desirable to apply chemical methods for this purpose, and also to estimate at the same time the phosphorus, so as to see how far the degenerative liberation of phosphoric acid determines or is associated with these variations. It is realized, however, that the chemical method has limitations, as there might be microscopically increased calcium sufficient to affect the activity of a small area of nervous system without there being any appreciable effect on an estimation of calcium in a large amount of tissue. An attempt is therefore made to combine the results of chemical and histological examination.

PROCEDURE AND METHODS.

In order to define if possible the nature of the combinations of calcium present in the normal brain, 100 gm. of fresh material was extracted first with hot water, then with strong acetic acid, and finally with boiling alcohol. The aqueous extract was obtained clear after acidification and re-filtering; the acetic acid extract was neutralized with ammonia and filtered; in the filtrates calcium was estimated. The alcoholic extract was evaporated to dryness,

incinerated, and calcium estimated in the ash, and the residual tissue was treated similarly. Calcium was estimated by precipitation as oxalate and subsequent titration with potassium permanganate.

After filtering off the calcium oxalate in the first two extracts the phosphate was precipitated with ammonium magnesium citrate in excess of ammonia, the precipitate converted into pyrophosphate and weighed.

The results of the analysis are given in Table I.

TABLE I.—*Distribution of Calcium and Phosphorus in Fresh Normal Brain; mgm. per 100 gm.*

	Calcium (Ca).	Phosphorus (P).
In aqueous extract	0·2	4·15
In acetic acid extract	1·2	17·3
In alcoholic extract	0·7	Not estimated.
In residual tissue	1·05	,,
Total	3·15	

It follows that there is but a trace of calcium in the form of salts soluble in water; more than a third of the whole is extracted by acetic acid, and in this should be any calcium present originally as phosphate or carbonate, and probably most of that as soap, if any soap was there, though some of this would perhaps be contained in the alcoholic extract.

The amount of phosphoric acid extracted by acetic acid is greatly in excess of that which corresponds to the calcium so extracted. Its origin was not determined, but it was proved that no free phosphoric acid is split off from lecithin (a preparation made by Levene's method by Prof. Leathes) by the treatment, although an organic phosphoric acid, soluble in water, is presumably the glyceryl ester, and amounts to about one-third of the total phosphorus of the lecithin.

But the quantities of the fractions and the indefinite nature of the fractionation made it unprofitable to attempt anything but the estimation of the total calcium and phosphorus in the tissues.

The material was from human brains that had been kept some months in formalin. For some days the brain gains in weight when placed in formalin, up to nearly 7 per cent., but the weight decreases again, and by the end of two months the weight is almost back to that of the fresh tissue. The formalin contains traces of calcium extracted from the tissue, but in amounts too small to affect the results. So, too, with the phosphorus; Weil (1929) estimates that as much as 10 per cent. of the phosphorus in the cortex is thus lost in the fixing fluid. Wherever either calcium or phosphorus was found to be low in the analyses here recorded, the amounts present in the fixing fluid were determined, but the result showed that the low figures were not accounted for by increased amounts being taken up by it.

TABLE II.

	Case and duration of disease.	Nervous disease.	Age.	Remarks.	Amount of calcium (Ca) in milligrammes in 100 gm. of cerebral cortex.	Size of brain.	Amount of phosphorus (P.) in grammes in 100 gm. of cerebral cortex.
1	... ...	Normal	5	...	3·8	43½ oz.	0·153
2	... (M.)	Renal infantilism	16	...	3·2	Normal size	0 159
3	... ...	Normal	49	Renal abscess	5·8	„ „	...
4	... (M.)	Myasthenia gravis	29	...	5·3	...	0·179
5	E. G—. (F.)	Arterio-sclerotic insanity	72	...	6·0	...	0·112
6	R—. (M.)	„ „	80	...	5·4	43 oz.	0 104
7	B. C—. (M.)	„ „	80	Insane for 37 years	5·0 (basal ganglia) 4·7 (cortex) 6·9 (basal ganglia)	...	0·079
8	J. W. S—. (M.)	Cerebral tumour (ganglio-neuroma)	30	Portion of cortex a little distant from tumour analysed	7·8	48 oz.	0·201
9	W. L—. (M.) 2 years 4 months	Epidemic encephalitis	29	Diabetes insipidus, obesity, epilepsy, faint trace of Parkinsonism. Sudden death in epileptic fit	9·8 (frontal cortex)	48 oz. (no wasting)	0·171
10	G. E. R—. (M.) 3 years 8 months	„ „	50	Advanced Parkinsonism; cachexia	5·8	46 oz. (slight general wasting)	0·150
11	M. M—. (F.) 2½ years	„ „	53	Very advanced Parkinsonism. Extreme drowsiness for many months. Cachexia	5·6	44 oz. (slight localized wasting)	0·190
12	D. F. B—. (F.) 4 years	„ „	...	Epilepsy. Sudden death for which there was no apparent cause. Slight Parkinsonism mainly on one side	9·8	43 oz. (no wasting)	0·197
13	W. B—. (M.) More than 2 years	„ „	52	Drowsiness many months after onset of disease. Cachexia	5·7 (cerebral cortex) 6·7 (basal ganglia)	45 oz.	0·181
14	A. N—. (M.) 4 years	„ „	26	Advanced Parkinsonism	5·4	Weight not known, but brain rather small	0·122
15	G. F. G—. (M.) 2 years	„ „	31	Obesity. Epileptic before onset of epidemic encephalitis. Very advanced Parkinsonism	3·1	49 oz.	0·188
16	G. F. H |. (M.) 2½ years	„ „	32	Very advanced Parkinsonism. Death during malaria used as an experimental therapeutic measure	...	50 oz.	0·174

17	A. E. S—. (F.) 5 years	Epidemic encephalitis	62	Advanced Parkinsonism	...	43 oz.	0·174
18	F. E—. (M.) More than 10 years	Idiopathic epilepsy	29	*Status epilepticus*	4·3	No wasting	0·192
19	L. W—. (F.) 11 years	„ „	30	„ „	5·4	46 oz.	0·181
20	E. M—. (F)	Tuberose sclerosis; epilepsy	29	„ „	30·2	39¼ oz.	0·183
21	E. W—. (F.) 9 years	Epilepsy; mental deficiency; dyspituitarism	16	„ „	4·3	40 oz.	0·071
22	F. A—. (M.) 12 years	Epilepsy; pituitary infantilism; mental deficiency; hypertrophy of the brain	21	„ „	4·8	59 oz.	0·089
23	M. W—. (F.) 33 years	Epilepsy; mental deficiency	36	„ „	3·1	Small	0·087
24	G—. (M.)	General paralysis of the insane (acquired)	44	...	5·7	40 oz.	0·110
25	H—. (M.)	Ditto	47	...	4·4	46 oz.	0·105
26	J. B—. (M)	„	48	Inoculated with malaria one month previously	9·9	45 oz.	0·138
27	R. G. S—. (M.)	„	44	...	3 9	48 oz.	0·131
28	J—. (M.)	Congenital general paralysis of the insane	16	...	4·0	47 oz.	0 166
29	K. C. B—. (M.)	Ditto	25	...	4·3	43 oz.	0·147
30	L B—. (F.)	Huntington's chorea	43	Cases 30 and 31 were sisters	2·7	40 oz.	0·124
31	A. B—. (F.)	„ „	..	...	3·1	38 oz.	0·079
32	K—. (M.)	„ „	52	...	4·1	53 oz.	0·132
33	C. B—. (F.)	„ „	59	...	3·2	36 oz.	0·089
34	V. H—. (F.) From birth	Nervous cretinism	15	No thyroid present	7·7 (frontal cortex) 7·75 (occipital cortex)	36¼ oz.	0·056
35	F—. (M.) Duration uncertain	Multiple microscopic hæmorrhages in the brain were apparently the cause of death	26	Patient died a week after the onset of symptoms	19·8	...	0·209
36	A. H—. (F.)	Paget's disease	75	Skull very thickened (about 1 in. in places)	7·6 (occipital)	43 oz.	0·127
37	S—. (F.)	Insanity of long duration	75	...	10·8	Small sclerotic brain	0·208
38	V. W—. (F.)	Imbecility. (Cause of of death, phthisis.)	54	...	4·9	...	0·197
39	A. W—. (M.)	Acromegaly	...	...	4·8	59 oz. (approximately)	0·135

About 50 gm. of cortical tissue, cut in slices 3 or 4 mm. thick, so as to contain as little of the white matter as possible, were dried on filter-paper and then weighed. After drying in a steam oven the weighed quantity was heated in a silica crucible, with the addition of a little ammonium nitrate to facilitate the oxidation. The ash was taken up in hydrochloric acid, ammonia added and then acetic acid; a slight brownish precipitate that generally formed was filtered off. The calcium was precipitated as oxalate and the calcium oxalate estimated with N/100 potassium permanganate. The phosphate was precipitated with ammonium magnesium citrate in excess of ammonia, the precipitate converted into pyrophosphate and weighed. Usually two or more estimations were made.

Most of the brains used have been examined histologically; the majority of the cases I had myself had the opportunity of studying clinically at the South Yorkshire Mental Hospital. An account of some of the cases of chronic epidemic encephalitis has been already published (Eaves and Croll, 1930), also of the cretin, No. 34 (Eaves and Croll, 1928), and of one of the pituitary cases (Eaves, 1930).

Analyses of 35 brains from cases of disease of the central nervous system and 4 other cases for comparison are given in Table II.

RESULTS.

There are very few determinations of the amount of calcium in the brain on record for comparison. Geoghegan (1877) gives 4, without particulars as to the clinical condition; Moraczewski (1897) gives 6, none of them apparently from cases of nervous disease. These analyses, however, were all gravimetric; the volumetric method, more recently introduced, is far more suitable and trustworthy, and the recorded figures are some of them, very suspicious, varying in the latter paper from 2 mgm. to 90 mgm. per 100 gm. in brains that should have been normal. My figures for normal conditions of the brain range from rather more than 3 to less than 6 mgm. Ca per 100 gm. fresh weight. The amount tends to be near the higher limit with advancing age. Where, in a series of cases of one type, the figures are *all* near the lower limit or beyond it, it is reasonable to regard the calcium as abnormally low; where, in another type of case, the figures are *all* above 5 mgm., as abnormally high; a number of the analyses show results very considerably higher than this.

For phosphorus the range of variation is much less, and abnormal figures in the sense of deficiency rather than excess are the conspicuous ones. Figures lower than 0·140 gm. phosphorus per 100 gm. fresh weight are certainly significant; whether figures higher than 0·180 are it is difficult to say; in only 2 cases were figures higher than 0·197 found, the highest being 0·209.

In correlating the results given in the table, attention may be called to the following points:

(1) In 13 cases of *degenerative diseases*, arterio-sclerotic insanity (Cases 5 to 7), general paralysis (Cases 24 to 29), Huntington's chorea (Cases 30 to 33) the *phosphorus* is below the normal range of variation, except in the 2 cases of congenital general paralysis, in one of which the figure is near the middle

of the normal range. Loss of phosphorus compounds, as pointed out in the introduction, has for thirty years been recognized as characteristic of general paralysis. The 4 cases of Huntington's chorea are among the lowest (average 0·105, highest 0·132), and in connection with the suggestion above that degeneration of phospholipines may lead to liberation of phosphoric acid and deposition of calcium phosphate, it is remarkable that this disease is the only one, with perhaps one exception, in which the calcium figures are consistently low. In the 6 cases of general paralysis, with one exception, the calcium figures are normal; in the exception, a case treated with malaria, they were well beyond the upper limit of normality. In the 3 cases of arterio-sclerotic insanity they are just about or beyond the normal.

That the one condition here reported on, in which, as in tetany, the calcium is definitely diminished, should be Huntington's chorea, is interesting in view of the increased excitability of the cerebral cortex shown by the restlessness of patients with this disease.

(2) In the nine cases of *epidemic encephalitis* there was only one in which the phosphorus was low (No. 14, 0·122); in all the others it was near the upper limit, if not just beyond it, and in 3 ranged from 0·190 to 0·197. In 7 of the 9 calcium was estimated; in 2 it was well above the normal range (9·8), and in 4 at about the upper limit; in 1 near the lower limit. In the 2 brains with the largest amount of calcium (Nos. 9 and 12), which were those of comparatively young patients, there was nothing in the histological appearances to suggest increased calcium, although in No. 9 the blood-vessels appeared abnormal in the hypothalamus and brain-stem; both these patients died suddenly, the only apparent cause in No. 12 being multiple microscopic hæmorrhages in this region. In No. 13 an estimation of calcium was done in the basal ganglia as well as in the cortex; both results were high, the former especially so—6·7 mgm.; in this case there were also microscopic deposits in the midbrain which appeared to be calcium salts (Eaves, 1926). In No. 14 histological appearances also suggested increased calcium in the brain-stem and hypothalamus, although in the cortex the amount found by analysis was only slightly higher than the normal average for the age. The significance of increased calcium in relation to disorders of sleep is discussed by Eaves and Croll (1930).

It is remarkable that the extreme wasting in Cases 11 and 13, who were drowsy for many months before death, was compatible with normal brain weight and an amount of phosphorus close to the upper limit of the normal range. That the brain weight is maintained in starved animals is an old observation.

(3) *Epilepsy.*—All the 6 patients reported here died in *status epilepticus*. In 5 there was no abnormal amount of calcium. In 3 the phosphorus was also normal in spite of extensive chromatolysis; in the other 3, who had suffered from epilepsy from early childhood, who were mentally deficient, and showed definite signs of disordered pituitary function, the phosphorus was about 50 per cent. below normal figures. No. 22 exhibited pituitary infantilism and hypertrophy of the brain; in No. 21 there was an abnormal condition of growth and of the musculature. It is probable that the low phosphorus content of the brain may be correlated with the dyspituitarism and not directly with the epilepsy.

The one case in which the calcium was abnormally high was a case of tuberose sclerosis. Bertrand and Antschul (1927) say that calcium deposition is unusual in this condition, but this statement is based entirely on histological evidence. In Case No. 20, which had six or seven times the normal amount of calcium, although many methods were tried for detecting it, there was no indication from the microscopical appearances of the unusual amount beyond a rather diffuse patchy staining of the cortex with methylene-blue.

(4) *Tumours of the brain.*—Calcification is commonest in cerebral angiomata ; it occurs also in gliomas, but other cerebral tumours undergo necrosis without calcification. I found a high degree in 2 cases of angioma (Eaves, 1926), and have recently noted calcareous deposits in the neighbourhood of a gliomatous cyst of the cerebellum. In this series in No. 8, a ganglio-neuroma of the white matter, the calcium was high, 7·8 mgm. per 100 gm., in the cortex near by. Calcification of the nervous parenchyma in the neighbourhood of cerebral tumours, especially where there is intra-cranial compression, has been noted by Bertrand and Antschul (1927).

(5) *Cretinism.*—In the cretin's brain examined (No. 34) the calcium was remarkably high in both the frontal and the occipital lobes. This is possibly accounted for by the increase in calcium in the vessel walls, the purpurin reaction being positive in them and also in deposits in various parts of the brain, especially the corpus striatum. The very low figure for phosphorus is probably partly, but not altogether, due to the incomplete myelination of the nerve-fibres. An account of this case has been published (Eaves and Croll, 1928).

The case of *Paget's Disease*, No. 36, had an equally large amount of calcium in the cortex. The skull was very thick, and increased intra-cranial pressure may have been a factor determining high calcium content of the cortex.

(6) *Multiple hæmorrhages* and calcification in the brain (Case No. 35), where death resulted a week after the first symptoms, is remarkable. A full clinical report of this case has already appeared (Hall, 1929). There was about five times the normal amount of calcium in the cortex and many minute hæmorrhages, but no inorganic iron stainable by the Prussian blue reaction. The deposition of calcium had, therefore, been the primary event, and the hæmorrhages the secondary result. This was true also in Cases 9 and 12, where there were capillary hæmorrhages ; reference was made to these cases in the section on epidemic encephalitis.

DISCUSSION.

Poor myelination is always accompanied by a low phosphorus content of the cerebral cortex. On the other hand, the latter may be low where myelination (as shown by the Weigert-Pal method) is excellent. A good example of this is No. 22, to which reference has been made in a preceding paragraph. This nervous system seemed very well developed as far as could be ascertained from histological examination ; the brain weight was very high, and contrasted very much with the low degree of mentality of the patient. The poverty in phosphorus is evidently in the nerve-cells rather than the fibres. It is impossible to attribute the abnormal chemical composition only to an increased water content of the brain. Another example of high brain weight

and rather low phosphorus percentage is seen in acromegaly (No. 39), where, however, unlike the preceding case, the total quantity of phosphorus in the brain compared favourably with that in other brains.

In Nos. 8, 35 and 37 there is an association between high calcium and a slightly higher phosphorus content than normal. There was a gliosis in these brains, but it is difficult to suggest which is cause and which effect.

SUMMARY AND CONCLUSIONS.

1. An increase of calcium in the brain is relatively uncommon. It tends to occur in conditions where there is gliosis without wasting of the brain.

2. Increased calcium was found in the brains of two young patients in which numerous microscopic hæmorrhages were present and was not associated with the deposition of inorganic iron.

3. In 13 brains from cases of degenerative nervous diseases (general paralysis, Huntington's chorea, and arterio-sclerotic insanity), in all of which there was wasting, there was increased calcium only in 1 and great diminution in phosphorus compounds in 11. In the 4 brains from cases of Huntington's chorea the calcium was diminished.

4. In chronic epidemic encephalitis increase of calcium is more common than in other conditions. In 8 out of 9 cases the phosphorus percentage was within normal limits.

5. In epilepsy in 6 cases with *status epilepticus* the calcium was within normal limits except in one case of tuberose sclerosis. In 3 of the cases the phosphorus was within normal limits. In the other 3, in which epilepsy was associated with mental deficiency and dyspituitarism, the phosphorus was very low. This was probably due to disordered phosphorus metabolism in endocrine disorders rather than necessarily a result of *status epilepticus*.

6. In 1 case of cretinism the calcium was high and amount of phosphorus very low.

7. In 1 case of Paget's disease the amount of calcium was high.

8. A low phosphorus content may exist with good myelination, but poor myelination is always accompanied by a decreased amount of phosphorus. It is concluded that nerve-cells may pathologically be deficient in compounds of phosphorus, probably of a lipoid nature.

9. Abnormal function of the nervous system may be associated with chemical alterations in the brain, without necessarily any very definite histological changes.

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